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INTERACTION OF THE NUCLEOSIDE TRANSPORT INHIBITOR, NITROBENZYLTHIOINOSINE 5'-MONOPHOSPHATE, WITH HELA CELLS AND ERYTHROCYTES.

BY

© PATRICK OKAFOR JAMES OGBUNUDE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1979.

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "INTERACTION OF THE NUCLEOSIDE TRANSPORT INHIBITOR, NITROBENZYLTHIOINOSINE 5'-MONOPHOSPHATE, WITH HELA CELLS AND ERYTHROCYTES" submitted by PATRICK OKAFOR JAMES OGBUNUDE in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) is a potent and specific inhibitor of nucleoside transport by various animal cells. NBMPR binds with high affinity to specific membrane sites which are part of the transport mechanism; the occupation of these sites by NBMPR prevents transporter function. The present study demonstrated that NBMPR 5'-monophosphate (NBMPR-P), a potent inhibitor of nucleoside transport by HeLa cells, was not inhibitory *per se*, but became active after dephosphorylation.

That dephosphorylation of NBMPR-P was involved in inhibition of nucleoside transport by this compound became apparent in experiments which showed that (i) cells exposed to [^{35}S]NBMPR-P or [G-^{3}H]NBMPR-P retained the isotopic labels virtually exclusively in the form of NBMPR and (ii) erythrocytes of the mouse and of man which do not possess ecto-5'-nucleotidase activity did not bind ^{35}S or ^{3}H after incubation with the labelled NBMPR-P preparations, but did so from [^{35}S]NBMPR or [G-^{3}H]NBMPR.

It was concluded that the dephosphorylation site for NBMPR-P is separate from and functions independently of the high affinity binding sites for NBMPR in HeLa cells. Evidence supporting this conclusion was based on observations that the cleavage of NBMPR-P progressed in the presence of saturating concentrations of NBMPR (5 μM) at rates similar to those in the absence of NBMPR.

Presuming the validity of the conclusion that cell surface sites for NBMPR-P dephosphorylation and NBMPR binding are distinct, it would be expected that upon dephosphorylation of NBMPR-P and release into the medium of the product, NBMPR, the latter would (i) mix by diffusion with NBMPR already present in the medium and (ii) exchange with bound NBMPR. However, the initial rates of binding of ^{3}H from [G-^{3}H]NBMPR-P were not a function of the extracellular concentration of nonisotopic NBMPR, suggesting that to an appreciable extent the dephosphorylation product, [G-^{3}H]NBMPR, was bound before isotopic dilution took place.

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LIST OF ABBREVIATIONS

NBMPR	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (nitrobenzylthioinosine)
NBMPR-P	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine 5'-monophosphate (nitrobenzylthio-inosine 5'-monophosphate)
NBTGR	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribo-furanosyl purine (nitrobenzylthioguanosine)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Nebularine	9-β-D-Ribofuranosylpurine
Tubercidin	4-Amino-7-(β-D-ribofuranosyl)pyrollo[2,3 d] pyrimidine
Toyocamycin	4-Amino-5-cyano-7-(β-D-ribofuranosyl)-pyrollo[2,3 d]pyrimidine
Sangivamycin	4-Amino-5-carboxamide-7-(β-D-ribofuranosyl) pyrollo[2,3 d]pyrimidine
K_{dissoc}	Dissociation constant
E5Nase	Ecto-5'-nucleotidase
P	Phosphoryl group
H	Hypoxanthine
AR	Adenosine
AdR	2'-Deoxyadenosine

GR	Guanosine
GdR	2'-Deoxyguanosine
HR	Inosine
IMP	Inosinate
XMP	Xanthylate
sAMP	Adenylosuccinate
dAMP	2'-Deoxyadenosine monophosphate
dGMP	2'-Deoxyguanosine monophosphate
TLC	Thin-layer chromatography.

I INTRODUCTION

A. The Problem

NBMPR is an inhibitor of nucleoside transport that binds tightly but reversibly to the plasma membrane of animal cells (14,22,23,38,59). The 5'-monophosphate of this compound also is an inhibitor of nucleoside transport in HeLa cells with potency comparable to that of NBMPR (44). It is known that HeLa cells possess 5'-nucleotidase activity intrinsic to the plasma membrane and that this activity is oriented toward the external medium rather than toward the cell cytoplasm (7). The main objective of the study was to determine whether the transport inhibitory activity of NBMPR-P was attributable to NBMPR derived through dephosphorylation of NBMPR-P by the ecto-5'-nucleotidase. During the course of this investigation, it was observed that an anomaly existed in the initial binding kinetics of NBMPR-P and a possible explanation was sought in the relationship of the dephosphorylation sites to the NBMPR binding sites.

B. Literature Review

1. Nucleoside Transport

The transport of nucleosides across plasma membranes has been studied in many types of animal cells and has often been described as a facilitated diffusion process (11,42,54,60); however the characteristics of facilitated diffusion have been demonstrated in studies of nucleoside permeation only with cells which lack the ability to

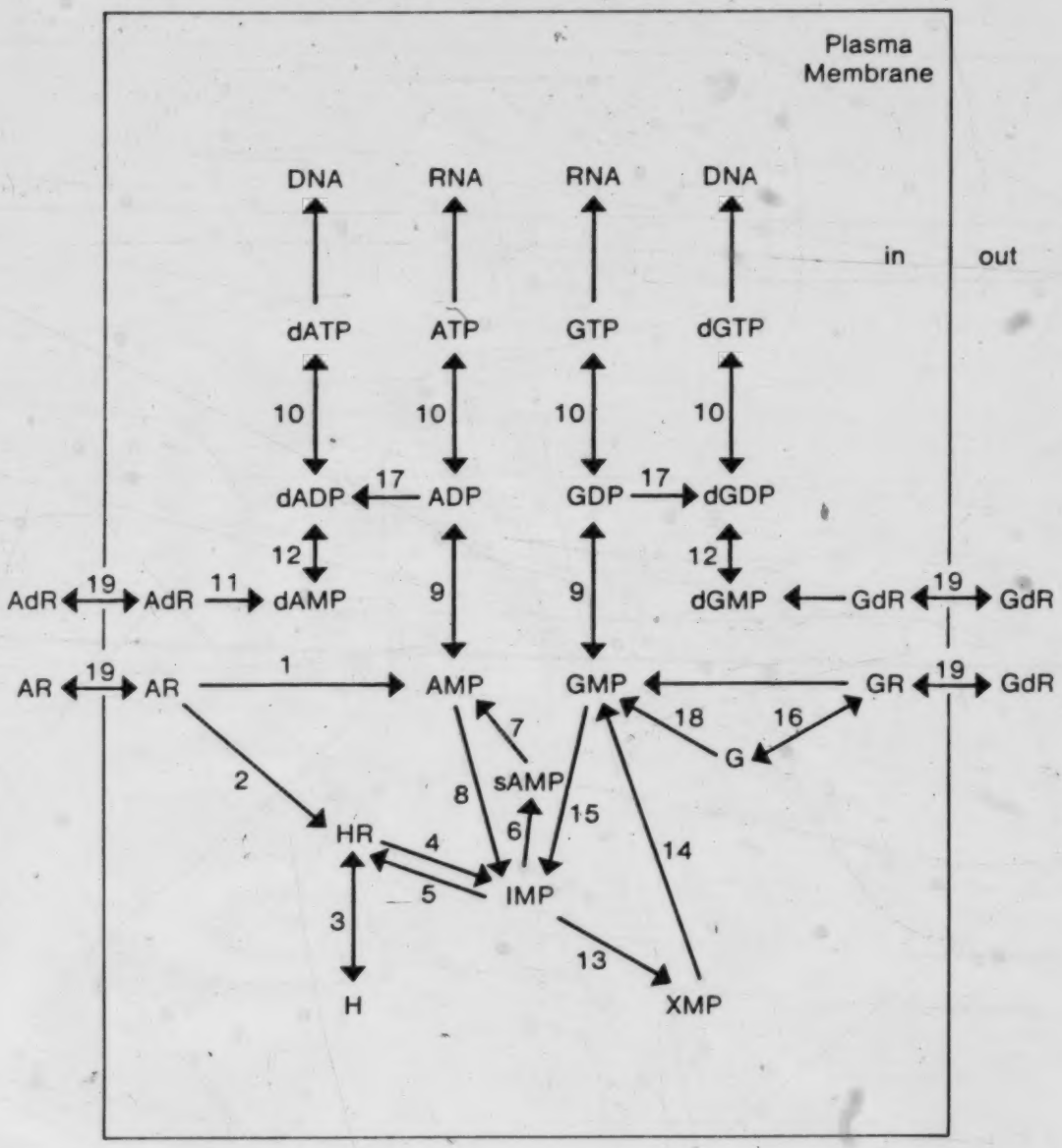
metabolize the permeant. Uridine and thymidine transport in human erythrocytes and in cells which are unable to metabolize either nucleoside have the characteristics of facilitated diffusion processes (11,12). In cells capable of metabolizing nucleosides, the transport of these compounds has been studied mainly through the kinetics of permeant uptake. Steady state rates of cellular nucleoside uptake are determined by the slowest of the events comprising the sequence of the uptake process.[1] In some cell-permeant systems, nucleoside phosphorylation appears to be rate-limiting in the uptake process (76). It is generally accepted that initial rates of nucleoside uptake provide a measure of the initial step (transport) in the uptake process. Thus, the study of nucleoside transport in cells which trap influent nucleosides as nucleotide metabolites has been approached mainly through study of the initial rate kinetics of nucleoside uptake.

The metabolism of purine nucleosides is illustrated in Fig. 1. ATP is the principal metabolite of adenosine in HeLa cells; some conversion of adenosine to inosine, hypoxanthine and inosinate also occurs (52).

---

[1]Overall, the process of nucleoside uptake involves transport of substrate across the cell membrane, phosphorylation by a nucleoside kinase, further phosphorylation by nucleoside monophosphate and diphosphate kinases, and other metabolic fates, including incorporation into nucleic acids.

Figure 1 Pathways of nucleoside metabolism in animal cell (31)

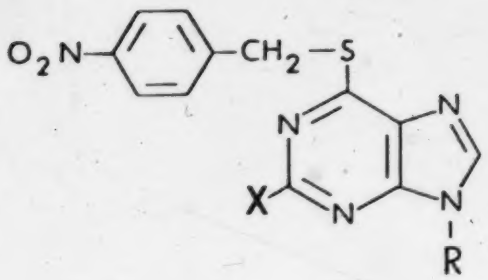


NOMENCLATURE OF ENZYMES OF PURINE METABOLISM

Enzyme Number	Common Name	Systematic Name	EC Number
1	Adenosine kinase	ATP:adenosine 5'-phosphotransferase	2.7.1.20
2	Adenosine deaminase	Adenosine aminohydrolase	3.5.4.4
3	Purine nucleoside phosphorylase	Purine nucleoside:ortho-phosphate ribosyltransferase	2.4.2.1
4	Inosine kinase	ATP:inosine 5'-phosphotransferase	2.7.1.73
5	5'-Nucleotidase	5'-Ribonucleotide phosphohydrolase	3.1.3.5
6	Adenylosuccinate synthetase	IMP:L-aspartate ligase (GDP)	6.3.4.4
7	Adenylosuccinate lyase	Adenylosuccinate:AMP lyase	4.3.2.2
8	AMP deaminase	AMP aminohydrolase	3.5.4.6
9	Nucleoside monophosphate kinase	ATP:nucleoside monophosphate phosphotransferase	2.7.4.4
10	Nucleoside diphosphate kinase	ATP:nucleoside diphosphate phosphotransferase	2.7.4.6
11	Deoxyadenosine kinase	ATP:deoxyadenosine 5'-phosphate transferase	2.7.1.76

12	Deoxynucleoside mono-phosphate kinase	ATP:deoxynucleoside mono-phosphate phosphotransferase	2.7.4.13
13	IMP dehydrogenase	IMP:NAD oxidoreductase	1.2.1.14
14	XMP aminase	XMP:L-glutamine amino-ligase	6.3.5.2
15	GMP reductase	Reduced NADP:GMP oxidoreduc-tase.	1.6.6.8
16	Guanosine phosphorylase	Guanosine:orthophosphate ribosyltransferase	2.4.2.15
17	Ribonucleoside-diphosphate reductase	2'-Deoxyribonucleoside-dipho-sphate oxidized-thioredoxin 2'-oxidoreductase	1.17.4.1
18	Hypoxanthine (Guanine) phosphoribosyltransferase	IMP pyrophosphorylase trans-phosphoribosidase	2.4.2.8
19	Nucleoside permease	-	-

Fig. 2 Structural formulae of the inhibitors.



Compound	Substituent	
	X	R
NBTGR	NH_2	Ribosyl
NBMPR	H	Ribosyl
NBMPR-P	H	Ribosyl 5'-phosphate

2. Inhibitors of Nucleoside Transport

a. Inhibitors. A number of compounds[2] chemically unrelated to nucleosides have been recognized as potent inhibitors of nucleoside transport (41,43,44,67,68). Of these. Persantin (42), cytochalasin B (45), phloretin (69) and colchicine (42) are nonspecific in that they also inhibit the transport of certain compounds other than nucleosides. Nitrobenzylthioinosine and various S^6-derivatives of 6-thioinosine and 6-thioguanosine are potent and specific inhibitors of nucleoside transport in a variety of animal cells (14,32,33,70), including HeLa cells (2,3,15). NBMPR is specific in that the uptake of bases, sugars or amino acids is not inhibited (10). As well the aglycone of NBMPR is inactive (8,46). The phosphorylation of uridine, thymidine or adenosine and the distribution of the metabolites of these compounds in extracts of cultured cells are not affected by NBMPR (2,10).

b. Binding of NBMPR Erythrocytes of the mouse and of man (14) and various lines of cultured animal cells[3] have been shown to possess binding sites for NBMPR. Two modes of interaction of NBMPR with cells have been

---

[2]Persantin (dipyridamole) (43), cytochalasin B (44), phloretin (67), aflatoxins (41) and p-chloromercuribenzoate (68).

[3]HeLa cells (15), erythrocytes (33,12), MCT hamster (32), Novikoff hepatoma, Chinese hamster ovary, Mouse L and P338 murine leukemia cells (70).

recognized: a saturable binding to high affinity sites and a nonsaturable binding of NBMPR proportional to its concentration in the medium (14). In erythrocytes, the high affinity binding sites were on the plasma membrane and binding was (i) reversible, (ii) involved no detectable chemical transformation of NBMPR (38,59) and (iii) correlated with the inhibition of nucleoside transport (13,22, 23,38,59,76). Similar observations have been made with HeLa cells (38 and the present study) except that the probable location of the NBMPR binding sites in the plasma membrane has not been shown. The number of high affinity binding sites for NBMPR on cells of various types has been determined (14,23,38,59,76). In HeLa cells, the occupancy of these sites by NBMPR correlated with the inhibition of the nucleoside uptake, however the NBMPR concentration-effect relationship was not simple, since at near total occupancy of these sites by NBMPR, 10-30% of nucleoside transport activity remained functional (14,23,38,76). Structure-activity studies in which NBMPR congeners were compared for their ability to inhibit nucleoside transport have identified features of the NBMPR molecule that contribute importantly in the interaction with the cellular binding site (51,58). The 9-pentosyl and 6-(4-nitrobenzyl)thio groups contribute importantly to the inhibitory activity. The 2'-hydroxyl group is not involved in the inhibitor-transporter interaction.

3'-O substituents are less tolerated than those at the 2'-O position. The β-anomer is preferred at the N^9-glycosidic bond.

3. Membrane Associated Enzymes

A number of membrane-bound enzymes are known in animal cells, some of these, for example, adenylate cyclase (EC 4.6.1.1) and $(Na^+K^+)Mg^{2+}$ATPase (EC 3.6.1.4) have their active sites on the inward side (cytoplasmic face) of the plasma membrane. Membrane enzymes with active sites that face only the external medium are termed "ecto-enzymes".

a. Ecto-enzymes A number of enzymes of nucleotide metabolism, for example, 5'-nucleotidase (EC 3.1.3.5), Mg^{2+}ATPase (EC 3.6.1.3), p-nitrophenylphosphatase (EC 3.1.3.1) and adenosine monophosphate deaminase (EC 3.5.4.6), are recognized as ecto-enzymes in several cell lines (7,17,27,34,46,64,68,73). Enzyme activities that are outward facing in one cell type may not be so oriented in other cells; for example, the apyrase (EC 3.6.1.5) activity of guinea pig liver cells is outward facing and is mainly cytoplasmic in two lines of cultured cells, Chang liver cells and KB cells (69). As well, the acetylcholinesterase of erythrocytes is recognized as an ecto-enzyme, but this activity is not present on the external surfaces of leukocytes or muscle cells of the guinea pig (18,30).

b. Ecto-5'-Nucleotidase The presence of ecto-5'-nucleotidase activity has been demonstrated in a number

of cultured cells lines (17,46,68,73), including HeLa cells (7). The enzyme is inhibited by various divalent cations, notably, Cu^{2+}, Ni^{2+} and Zn^{2+} (32,66). At pH 7.4, the enzyme is not inhibited or stimulated by divalent cations (67). It is believed that a tightly-bound divalent cation is involved in the structure of the active enzyme (17). The enzyme dephosphorylates 5'-nucleotides, however, the order of attack (that is, the ability of the enzyme to dephosphorylate one nucleotide substrate relative to another) depends on the source of the enzyme. For example, AMP is a better substrate than UMP for the enzyme from leukocytes, while the reverse is true for the liver ecto-5'-nucleotidase (17,67). The enzyme is susceptible to inhibition by nucleoside di- and tri- phosphates (3,17). There are marked species differences in cardiac 5'-nucleotidase activity, rat hearts containing 100 times the specific activity found in rabbit heart (3).

The physiological significance of the ecto-5'-nucleotidase activity is obscure because nucleotides are not found in extracellular fluids *in vivo*. The observation, however, that the cardiac adenosine level is increased in response to oxygen insufficiency has led to the "adenosine hypothesis" of coronary flow regulation. Berne (5) proposed that in hypoxia, curtailment of ATP generation, leads to accumulation of its degradation products, ADP, AMP and adenosine. The adenosine so produced is released into the interstitial space of the heart, diffuses to the coronary

arterioles and, being a potent vasodilator, causes them to relax, thereby increasing coronary flow and oxygen delivery. However, this hypothesis does not explain how adenosine production is adjusted to the oxygen requirements of the heart. One approach to the latter problem was that employed by Baer et al. (3) who studied the regulation of adenosine production by cardiac 5'-nucleotidase. These investigators found that ATP inhibited 5'-nucleotidase activity and suggested this inhibition might contribute to the regulation of the ecto-enzyme. An arguement against this mechanism is the observation that ATP levels in heart do not change for at least 15 sec after coronary ligation, whereas adenosine levels increase in less than 5 sec (8,72). This account of the hypothetical role of adenosine in the regulation of coronary flow is intended only as a glimpse at an as yet unresolved issue that is part of the literature concerning the ecto-5'-nucleotidase activity of animal cells.

More recently, Frick and Lowenstein (29) have proposed that, in addition to the dephosphorylation of 5'-nucleotides the ecto-5'-nucleotidase may also participate in the vectorial transport of derived nucleosides into rat heart.

c. Ecto-ATPase Ecto-ATPase activity is found in a variety of cells (16,25,74,75), including human erythrocytes (65), HeLa cells (7), hepatocytes (34) and cells of the Ehrlich ascites carcinoma (64). The enzyme is stimulated by divalent cations, particularly by Mg^{2+}, and in some cells (for example, in human erythrocytes) it is inhibited by Ca^{2+}

(65). The ecto-ATPase is sensitive to monovalent cations and oubain (70). Agren et al. (1) showed that this enzyme hydrolyses the γ-phosphoryl group of ATP, releasing the inorganic phosphate into the medium. The enzyme also hydrolyses ADP and p-nitrophenylphosphate. The localization of the enzyme activity appeared to change with induction of neoplasia in liver cells, for example, the ATPase of normal rat hepatocytes was found localized on plasma membranes, facing bile canaliculi; in contrast, an intense activity was demonstrated over the entire surface of hepatocytes from rats fed with carcinogens (33).

II. MATERIAL AND METHODS

A. Chemicals

[G-^{3}H]NBMPR and [G-^{3}H]NBMPR-P were purchased from Moravek Biochemicals, City of Industry, Calif.; specific activities were 25 and 7 Ci/mmole, respectively. Cell culture materials were obtained from Grand Island Biological Co. Calgary, Alta. Snake venom 5'-nucleotidase was purchased from Sigma Chemical Co., St. Louis, Mo. The medium (MEM-S) for spinner and Vibro-Mixer suspension cultures consisted of Eagle's minimal essential medium (MEM) without calcium salts and supplemented with 5% calf-serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM HEPES (pH 7.4). Growth medium for maintenance of monolayer cultures (MEM-M) was MEM supplemented with 10% calf serum and 2 mM HEPES (pH 7.4). The medium employed in transport and binding experiments (MEM-T) consisted of bicarbonate-free MEM supplemented with 2 mM HEPES (pH 7.4) and 12 mM NaCl. NBMPR and NBTGR were prepared by established methods (49), using thioinosine and thioguanosine generously provided by the Division of Cancer treatment, National Cancer Institute Bethesda, Md. Dr. T.P. Lynch of this laboratory prepared NBMPR-P (44).

B. Cell-Culture

1. HeLa Cell Primary Stock Line

HeLa cell stocks[4] were stored in liquid nitrogen

[4]Mycoplasma-free, as determined by Dr. Janet Robertson, Department of Medical Bacteriology.

in antibiotic-free growth medium containing 10% dimethyl sulfoxide at a density of 10^7 cells per ml. Every eighth week, cultures were restarted from the frozen stock as follows. A vial was thawed at 37°, cells were collected by centrifugation (150 x g, 5 min) and resuspended in MEM.-A medium. Four 8 oz prescription bottles (Sani-Glas Ovals, Brockway Glass, Brockway, Pa.) were inoculated (10^5 cells per bottle) and incubated in a humidified CO_2 incubator at 37°.

Cells from trypsinized monolayer cultures were recultured weekly, establishing both spinner cultures and new monolayer cultures. The cultures were incubated at 37° in a humidified atmosphere of air - 5% CO_2 and cell concentrations in the spinner cultures were kept below 6 x 10^5 cells/ml. Under these conditions, cell proliferation was exponential with doubling times of 16 - 18 hr.

Cells from spinner suspension cultures were transferred to 2- or 5-litre round-bottom flasks in which cultures were kept under continuos agitation with vibrating mixers (Vibro-Mixer, Model E1, Chemapec, Hoboken, N.J.); such cultures were kept in exponential growth by dilution. The vibro-Mixer cultures were single-cell suspensions, in contrast to spinner cultures in which cells were associated in aggregates of several cells; for this reason, Vibro-Mixer cultures were used to prepare the inocula for replicate monolayer cultures (see below).

2. Replicate Monolayer Cultures

Replicate monolayer cultures were grown in 2 oz or 8 oz prescription bottles. Bottles were first "conditioned" with MEM medium supplemented with 20% calf serum for 24 hr at 37°. The conditioning medium was discarded and the bottles inoculated with 1.6 x 10^6 cells in 4.0 ml or 3.2 x 10^6 cells in 8.0 ml of MEM-S medium with final concentrations of calf serum and calcium salts adjusted to 7.5% and 0.9 mM and were incubated at 37° in a humidified atmosphere of 5% - CO_2 in air. The replicate cultures were used 24 hr after inoculation, at which time proliferation was exponential and cell numbers in the monolayers were close to those in the inoculum.

C. Binding Assay

1. Characteristics of NBMPR and NBMPR-P Binding to HeLa Cells

a. Binding Assays With HeLa Monolayer Cells The site-specific binding of NBMPR was determined from the cellular content of isotope following incubation of replicate monolayer cultures with [G-^{3}H]NBMPR, [^{35}S]NBMPR or their 5'-phosphate derivatives in the presence and absence of 5 µM NBTGR.

Thirty min prior to the start of binding assays, replicate monolayer cultures (with caps tightly closed to retain the CO_2 atmosphere) were transferred to the room in which the assay was to be conducted to allow for temperature equilibration and the cell number per bottle was determined

as the mean of 8 sample cultures. In determining the cellular content of bound NBMPR, culture bottles were processed individually, as follows. Growth medium was removed a few seconds before the assay, the bottle laid flat with the cell sheet uppermost, and the assay solution added (4.0 ml for 2 oz cultures, or 8.0 ml for 8 oz bottles). Unless otherwise specified, assays were performed with cultures in 2 oz prescription bottles. Binding intervals were initiated by rapid immersion of the cell sheet, accomplished by rotating the bottle 180° about its long axis. Five sec prior to the end of the assay interval, the medium was removed by suction and to end the interval, the cell sheet was flooded with ice-cold 0.15 M NaCl solution, filling the culture bottle; after 15 sec, the bottle was drained. For assay of the cellular content of ^{3}H or ^{35}S, 2.0 ml of 0.5 N KOH was added to each bottle and, after 45 min with occassional rocking, the content of each bottle was transferred to a scintillation vial by rinsing with four 4.0 ml portions of Tritosol phosphor solution (50), and the ^{3}H or ^{35}S activity determined by liquid scintillation counting. All assays were performed at least in duplicate.

b. Displacement of Bound NBMPR from HeLa Cells The displacement of bound [^{35}S]NBMPR or [G-^{3}H]NBMPR from HeLa monolayer cells was demonstrated in the following way. Replicate monolayer cultures (in triplicate) were exposed to MEM-T medium containing 5 nM [^{35}S]NBMPR or [G-^{3}H]NBMPR in

the absence and presence of 5 μM nonisotopic NBMPR. The reduction in the cellular content of ^{35}S or ^{3}H due to the presence of NBMPR measured site-specific binding of NBMPR. Intervals of exposure to [^{35}S]NBMPR or [G-^{3}H]NBMPR were terminated by removing the assay medium and washing the monolayers once with ice-cold NaCl solution prior to assay of their ^{35}S or ^{3}H content by the above procedure. Time courses of binding were obtained by varying the interval of exposure to [^{35}S]NBMPR or [G-^{3}H]NBMPR. Time courses of displacement were obtained by the addition of nonisotopic NBMPR or NBTGR (final concentrations 0.5 - 2.5 μM) to incubation mixtures in which binding was complete.

c. Ecto-5'-Nucleotidase Activity of HeLa Cells The rates at which HeLa cells converted extracellular [G-^{3}H]NBMPR-P to extracellular [G-^{3}H]NBMPR provided a measure of the cellular ecto-5'-nucleotidase activity; such assays were performed in the presence of 5 μM nonisotopic NBMPR to reduce cellular retention of the dephosphorylation product, [G-^{3}H]NBMPR to insignificant levels (see Sec. C.1. b.). No evidence has been obtained in this laboratory that NBMPR metabolites are formed in HeLa cells or that the NBMPR enters the HeLa cells (A.R.P. Paterson, personal communication).

Replicate monolayer cultures were washed twice with MEM-T medium and then incubated at 22° for 10 min with MEM-T medium containing 5 μM nonisotopic NBMPR before assaying rates of [G-^{3}H]NBMPR-P cleavage. Such assays

employed 4.0 ml portions of MEM-T medium containing various concentrations of [G-^{3}H]NBMPR-P and 5 μM NBMPR. Product formation was determined by TLC analysis of measured volumes of incubation medium; the fractions of the applied ^{3}H which accompanied carrier spots of NBMPR and NBMPR-P were determined by a combustion-liquid scintillation procedure. Time courses of product formation extrapolated through zero time and were constant for at least 20 min at the lowest substrate concentrations employed. Rates of ecto-5'-nucleotidase activity were determined from product formation during 10 min incubation intervals.

d. Identity of Bound ^{3}H After Incubation of HeLa Cells with [G-^{3}H]Nitrobenzylthioinosine and its 5'-Monophosphate The ^{3}H-labelled material which became associated with HeLa monolayer cells during incubation with [G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P was recovered from cells by methanol extraction and identified by thin-layer chromatography (TLC).

Extraction Monolayer cultures (8 oz bottles, about 3.2 x 10^6 cells each) were incubated in MEM-T medium containing [G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P under specified conditions, washed once with ice-cold NaCl solution and each extracted for 2 min with 3 ml portions of warm methanol. The extractions were repeated twice more and the three extracts from each culture were combined and concentrated to a small volume at 54° under a stream of nitrogen; 0.5 nmole portions of NBMPR and NBMPR-P (TLC carriers) were added to each

extract which was then dried under a stream of nitrogen at 54°.

Chromatography The extracts were dissolved in 100 µl of methanol and a 40 µl portion from each was applied (four 10 µl applications) as a 5 cm streak at the origin of a cellulose thin-layer chromatographic sheet (Eastman 6065, Eastman Kodak Co., Rochester, N.Y.) and developed in either solvent I, II or III (Table 1). NBMPR and NBMPR-P carrier zones were marked under UV light, scraped onto 5 x 5 cm pieces of Whatman No. 1 filter paper which were folded and combusted in a Packard Model 306 Sample Oxidizer in preparation for ^{3}H assay.

2. Binding Assay with HeLa Cells in Suspension

Cells from exponentially proliferating spinner cultures were collected by centrifugation (150 x g, 5 min), resuspended in MEM-T medium and the cell concentration adjusted to 0.3 - 0.4 x 10^{7} cells per ml. Binding assays at 22° were started by the addition of NBMPR or NBMPR-P labelled with ^{35}S or ^{3}H to cell suspensions in 50 ml conical tubes (1.0 - 2.0 x 10^{7} cells in 3.0 ml, final concentration) Intervals of binding were ended by centrifugation (500 x g, 3 min) and the supernatant fractions reserved for assay of ^{35}S or ^{3}H-content. Cell pellets were dissolved in 2.0 ml of 0.5 N KOH and assayed for ^{35}S- or ^{3}H-content by liquid scintillation counting using the xylene detergent-scintillant (50). The ^{35}S- or ^{3}H-content of the supernatant fluid was determined as described earlier (Sec.C.1.c.).

TABLE 1

Relative mobility (R_f) on thin-layer chromatograms

NBMPR and related compounds in 5-nmole portions were chromatographed on cellulose thin-layer sheets (Eastman 6065) using these solvents:

I n-Butanol:ethanol:water (4:1:2, v/v/v)

II Isoamyl alcohol saturated with 5% disodium hydrogen phosphate (1:1, v/v)

III 95% Ethanol:1 M ammonium acetate (7:3, v/v)

Compound	R_f in these solvents:		
	I	II	III
6-Thioinosine	0.47	0.87	0.55
6-Mercaptopurine	0.48	0.63	0.54
6-Thioinosinate	0.07	0.94	0.05
NBMPR	0.91	0.38	0.89
NBMPR-P	0.54	0.71	0.42

3. Binding Assay with Erythrocytes

a. Blood Collection Human blood was collected by venipuncture into evacuated heparinized tubes. Mouse blood, obtained by decapitation of 5 - 10 mice, was collected in cold phosphate buffered saline (PBS) (20) containing heparin (0.015 mg per ml). Erythrocytes were collected by centrifugation (1000 x g, 15 min), buffy coat fractions were discarded and, after two washes, were resuspended in an equal volume of cold PBS; cell concentrations were determined with samples of the same volume used in binding assays.

b. Binding Assays Binding assays with erythrocytes were performed with cell concentrations sufficiently high that cellular binding reduced the ligand content of the medium; such depletion measured cellular uptake binding of the ligand. In contrast, the practicalities of binding assays with cultured cells (monolayer cultures, in particular) required the use of much smaller numbers of cells per assay mixture and, hence, with cultured cells, determination of cell-bound ligand afforded a facile measure of binding.

To assay mixtures containing known concentrations of erythrocytes (about 0.9×10^9 cells per ml), [G-^{3}H]NBMPR was added, achieving final concentrations of 0.5 - 10.0 nM; assay mixtures were prepared with and without 5 µM non-isotopic NBTGR (a concentration sufficient to eliminate detectable, site-specific binding of 10 nM [G-^{3}H]NBMPR).

After incubation at 22° for 30 min, an interval sufficient to allow for equilibration of bound and free [G-^{3}H]NBMPR (P.M. Moore and A.R.P. Paterson, unpublished results), cells were pelleted and the ^{3}H-content of the supernatant determined. The extent to which the latter was reduced in the presence of nonisotopic NBMPR represented the cellular binding of [G-^{3}H]NBMPR.

III SYNTHESIS OF [^{35}S]NITROBENZYLTHIOINOSINE 5'-MONOPHOSPHATE

A. Introduction

The introduction of ^{35}S by exchange labelling with [^{35}S]elemental sulfur (48), a valuable means of labelling purine 6-thiones (40), was employed previously in this laboratory in the preparation of [^{35}S]NBMPR (38). In that procedure, 6-thioinosine was labelled by exchange and then alkylated in high yield with 4-nitrobenzyl bromide. During the present study, that procedure was adapted to the preparation of [^{35}S]NBMPR-P by Dr. P.M. Moore and Dr. T.P. Lynch of this laboratory. 6-Thioinosinate was labelled by exchange with [^{35}S]elemental sulfur and alkylated with 4-nitrobenzyl bromide.

B. Materials

6-Thioinosinate was prepared by Dr.T.P. Lynch of this laboratory (44). ^{35}S-Elemental sulfur (0.6 Ci/milliatom) was purchased from Amersham/Searle, Oakville, Ont.

C. Procedure

Described below is a procedure carried out cooperatively with Dr. P.M. Moore. The exchange reaction between 6-thioinosinate (500 nmole) and 1658 nmole of [^{35}S]elemental sulfur in 7 ml of dry freshly distilled pyridine was carried out in a vessel equiped with reflux condenser. The air in the system was displaced by nitrogen and the mixture was heated in an oil bath at 130° for 3 hr. After

the exchange reaction, the pyridine was removed by evaporation under a stream of nitrogen and 4-nitrobenzyl bromide (2 μmole) in 0.5 ml dry freshly distilled dimethylformamide was then added to the reaction vessel. The alkylation reaction was allowed to proceed at room temperature for 2 hr. After the reaction, approximately 50% of the dimethylformamide was driven off by evaporation under a stream of nitrogen.

The product was isolated by TLC as follows. The concentrated reaction mixture was applied as 12 cm streaks on thin layer silica gel plates prepared by spreading silica gel paste (Silica Gel (type 60); Merck, Darmstadt, Germany) on 20 x 20 cm glass plates in a 250 μm thick layer. The plates were washed with 15% methanol prior to use. Elemental sulfur moved near the solvent front and NBMPR-P remained at the origin. The product-containing band (located by UV light) was scraped into a small sintered glass funnel, extracted with five 1-ml portions of water and the extract was freeze-dried. The product was redissolved in 0.5 ml water and applied as 12 cm streaks on cellulose TLC plates. In preparing the latter, cellulose powder (Cellulosepulver MN 300, Macherey Nagel and Co., Duren, Germany) was homogenized with distilled water in a pestle homogenizer and the resulting paste was spread on 20 x 20 cm glass plates in a 250 μm thick layer; just prior to use the plates were washed with n-butanol:ethanol:water (4:1:2, v/v/v). The [^{35}S]NBMPR-P chromatograms were

developed in solvent I (Table 1); the product-containing band was scraped into a sintered glass funnel and the product eluted therefrom with water was stored in 50% methanol at -20°. Overall yield of [^{35}S]NBMPR-P was 75% in terms of 6-thioinosinate and the specific activity was 2.7×10^8 cpm per µmole.

D. Characterization of the Product

The radiochemical purity of the product was over 95%, as determined by chromatography on cellulose thin layers developed in solvents I and II. Chromatogram lanes were cut transversely into 1 cm segments which were assayed directly for ^{35}S-activity by liquid scintillation counting using xylene-detergent flour (50).

Further characterization of the compound was performed with snake venom 5'-nucleotidase. A mixture containing 0.049 M glycine-NaOH buffer (pH 8.5), 0.024 M $MgCl_2$, 0.017 µM [^{35}S]NBMPR-P and 0.01 mg enzyme in a total volume of 0.41 ml was incubated for 10 min at 37°. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 5%. Samples of the deproteinized mixture were analysed by chromatography on cellulose thin layers in solvents I and II (Table 1). More than 95% of the substrate was converted to [^{35}S]NBMPR, indicating that the compound was the 5'-monophosphate derivative of NBMPR.

IV RESULTS

A. Binding Characteristics of NBMPR and NBMPR-P

1. Efficiencies of the Extraction and Isolation Steps of the NBMPR Binding Assay

The determination of cell-associated ^3H derived from exposure of HeLa cells to [G-^3H]NBMPR or [G-^3H]NBMPR-P involved two steps: (i) methanol extraction and (ii) chromatographic isolation of the ligand from the extract. The efficiencies of these processes, performed as in routine binding assays, were determined in the experiments of Tables 2 and 3. The data of Table 2 indicate that the methanol extraction procedure was essentially quantitative; the 2-3% of cellular ^3H not extracted approximated the nonspecific[5] retention of ligand (that which occurred in the presence of 5 μM NBMPR).

The results shown in Table 3 illustrate the efficiency of the chromatographic isolation of NBMPR from methanolic extracts of HeLa monolayers exposed to [G-^3H]NBMPR. Recoveries in the extraction procedure(including evaporation of the methanol, drying, reconstitution, etc) were about 97%. About 91% of the original cellular

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[5]Specifically bound NBMPR is defined as the difference between the cellular content of labelled NBMPR in the presence and absence of 5 μM NBMPR (or NBTGR); the latter concentration is about 10^4 times higher than the dissociation constant of NBMPR at the specific binding sites (38).

TABLE 2

Recovery of cell-bound NBMPR
and NBMPR-P by extraction with methanol

Replicate monolayer cultures of HeLa cells in 8 oz prescription bottles were incubated at 22° for 20 min in MEM-T medium containing 5 nM [G-^{3}H]NBMPR or 5 nM [G-^{3}H]-NBMPR-P without (Groups A and B) or with (Group C) 5 μM nonisotopic NBMPR and then were washed once with ice-cold 0.15 M NaCl solution. Groups A and C: KOH digests of the cell sheets were assayed for ^{3}H content using the Tritosol scintillant. Group B: monolayers were extracted three times with methanol before assay of ^{3}H content in the same way. The data presented are averages of quadruplicate determinations.

Groups	Extracted	Cellular content of ligand (pmoles/10^6 cells)	
		[G-^{3}H]NBMPR	[G-^{3}H]NBMPR-P
A	No	0.41	0.37
B	Yes	0.01	0.008
C	No	0.01	0.007

TABLE 3

Efficiency of NBMPR isolation from methanolic extracts of HeLa cells

Replicate monolayer cultures of HeLa cells in 8 oz prescription bottles were incubated at 22° for 20 min in MEM-T medium containing 10 nM [G-^{3}H]NBMPR and were washed once with ice-cold 0.15 M NaCl solution. Group A monolayers were assayed directly for ^{3}H content. Group B monolayers were extracted with methanol, as in the NBMPR binding assay, and measured fractions of the extracted material were assayed for ^{3}H content. The monolayers of the extracted material were chromatographed on cellulose thin layers with NBMPR carrier; the ^{3}H content of the NBMPR zones was determined by the combustion method. Values listed are the means of triplicate determinations.

Group	Procedure	Cellular content of ligand	
		cpm/10^6 cells	Recovery (% of unextracted cpm)
A	Unextracted (KOH digest)	5171 ± 221*	100
B	Total extract	5037 ± 248	97.4 ± 1.3
C	Chromatographic isolate (NBMPR)	4722 ± 300	91.4 ± 2.1

*Average deviation from the mean.

content of ^{3}H was accounted for as chromatographically isolated NBMPR. Preliminary experiments showed that the sum of combustion assays representing entire chromatogram lanes accounted for 100 - 105% of the radioactivity applied to chromatograms.

The near-quantitative recoveries of cell-bound NBMPR demonstrated in the experiments of Tables 2 and 3 were the basis of the application of these procedures to the identification of the cell-associated material derived from NBMPR-P. It may be noted that the cellular ^{3}H content resulting from the incubation of HeLa monolayers with [G-^{3}H]NBMPR-P in the experiment of Table 3 was virtually entirely in the form of the dephosphorylation product, NBMPR. Similar results were obtained with HeLa cells in suspension following exposure to [G-^{3}H]NBMPR-P.

2. Determination of the Number of Nitrobenzylthioinosine Binding Sites per HeLa Monolayer Cell

The time course of NBMPR binding to HeLa cells at concentrations in excess of 8 nM is rapid, with the achievement of maximum values for the cell content of specifically bound NBMPR within 1 - 2 min (38). At lower concentrations of NBMPR which do not saturate the NBMPR binding sites, the time taken for equilibrium to be established between bound and free NBMPR is much longer; for example, when HeLa monolayers were incubated at 20° with [G-^{3}H]NBMPR, initially at 1.0 nM, about 30 min was required for the cell content of NBMPR to reach maximum values (E.Y. Lau and A.R.P. Paterson,

unpublished results). It is seen in Fig. 3 that the time course of [G-^{3}H]NBMPR-P binding was similar under the same conditions. This interval was employed in subsequent binding experiments with NBMPR-P.

The binding of ^{3}H-labelled NBMPR and NBMPR-P by HeLa cells was compared in the experiment of Fig. 4. In the presence of 5 μM nonisotopic NBTGR, the cellular content of ^{3}H was greatly reduced. NBMPR-specific binding sites were evidently occupied by NBTGR because the latter's concentration was 500 to 5000-fold higher than that of NBMPR. The difference between [G-^{3}H]NBMPR binding in the presence and absence of NBTGR is defined here as a measure of site specific-binding of NBMPR. It is seen in Fig. 4 that (i) NBTGR occupancy of the NBMPR binding sites precluded the binding of ^{3}H from [G-^{3}H]NBMPR-P, and (ii) at saturation, the cell content (NBMPR equivalent) of ^{3}H from that source was similar to that of NBMPR, suggesting that the bound form of NBMPR-P is the dephosphorylation product, NBMPR.

The effect of ligand concentration on the site-specific binding to HeLa cells of [^{35}S]NBMPR and [^{35}S]NBMPR-P was examined in the experiments summarized in Table 4. In the case of [^{35}S]NBMPR binding, the ^{35}S-content of the medium afforded a measure of the concentration of ligand in equilibrium with bound [^{35}S]NBMPR and mass law analysis of these data by the method of Scatchard (21) gave values for the maximum number of cellular binding sites and for the dissociation constant of NBMPR at those sites; these values

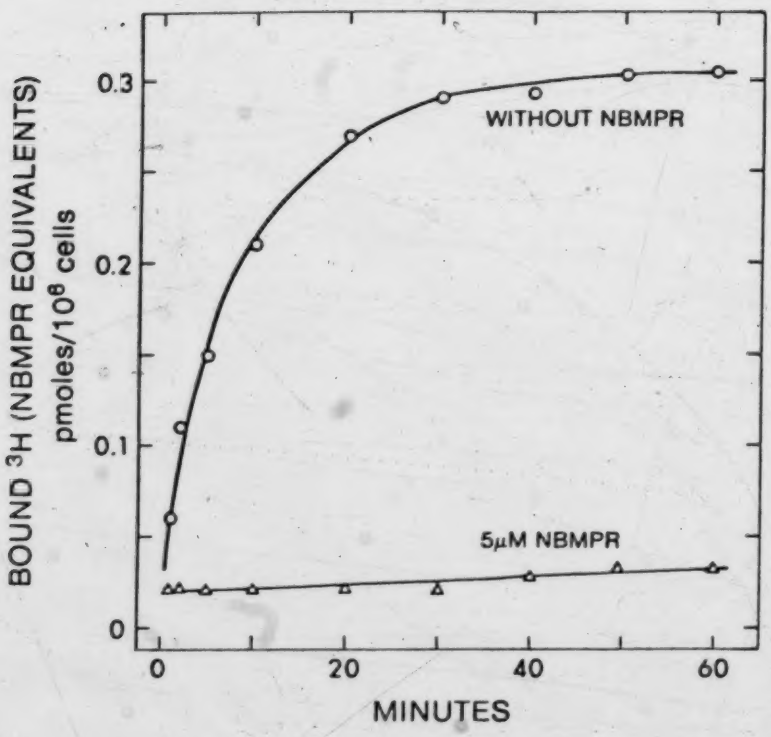

Fig. 3 Time course of NBMPR-P binding to HeLa cells

Replicate monolayer cultures were incubated at 22° for the indicated intervals in 4.0 ml MEM-T containing 1 nM [G-^{3}H]NBMPR-P with or without nonisotopic NBMPR 5 μM; the monolayers were then washed once with ice-cold 0.15 M NaCl solution and digested with KOH prior to assay of the cellular ^{3}H content with the Tritosol scintillant. The data shown are means of duplicate determinations.

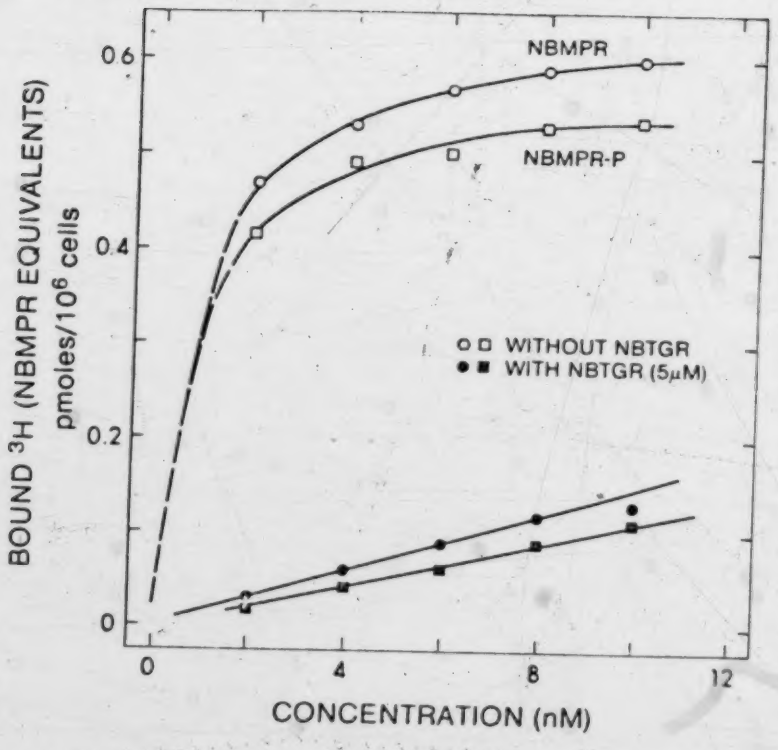

Fig. 4 Effect of concentration on binding of NBMPR-P and NBMPR to HeLa cells

Replicate monolayer cultures were incubated for 30 min at 22° in MEM-T medium containing [G-^{3}H]NBMPR (○) or [G-^{3}H]NBMPR-P (□) at the indicated concentrations with (● , ■) or without 5 μM nonisotopic NBTGR. Monolayers were washed once with ice-cold 0.15 M NaCl solution and cellular ^{3}H content determined by the KOH-Tritosol procedure.

TABLE 4

Binding of [^{35}S]NBMPR and
[^{35}S]NBMPR-P to HeLa monolayer cells

Replicate monolayer cultures were incubated for 30 min at 37°, 20°, 8° with MEM-T medium containing various concentrations (1.0 - 10.0 nM) of [^{35}S]NBMPR or [^{35}S]NBMPR-P with and without 5 µM nonisotopic NBTGR. Medium samples were reserved for determination of ^{35}S-content and monolayers were washed once with ice-cold 0.15 M NaCl solution prior to assay of ^{35}S-content by the KOH-Tritosol procedure. Binding data were subjected to mass law analysis by the method of Scatchard (21); the binding characteristics for NBMPR-P are recognized as approximations only for reasons given in the text (IV. A. 2.). The values in parenthesis have been reported (38).

Ligand	Assay temperature °C	Binding Characteristics	
		K_{dissoc} (M x 10^{-10})	Maximum number of molecules bound/cell x 10^{-5}
[^{35}S]NBMPR	37	1.3 (0.5)	3.2 (1.4)
	20	1.2 (1.4)	2.2 (1.0)
	8	1.3	1.0
[^{35}S]NBMPR-P	37	1.1	3.1
	20	0.9	1.7
	8	1.1	0.8

are 2 - 3 fold higher than previously reported values with the suspension cells (38). The reasons for these differences are not obvious. In the analysis of the data obtained when the binding substrate was [^{35}S]NBMPR-P, the assumption was made for the sake of arguement that the ^{35}S-content of the medium measured that of the ligand; however, it was recognized that the ligand might be either NBMPR, NBMPR-P or both. The similarity of the binding "constants" (Table 4) obtained in this way for NBMPR and NBMPR-P suggested that the latter compound was bound as NBMPR. Temperature change was observed to influence the binding of both compounds similarly, more binding sites being obtained at higher temperature than at lower temperature. The significance of the change in sites with temperature is not certain, however, the results are consistent with the idea that the cellular ligand in both cases was NBMPR[6]. It was not determined whether the differences in sites with change of temperature actually have any statistical significance.

The data of Tables 2 and 4 indicate that binding characteristics of NBMPR labelled with ^{35}S and ^{3}H were similar suggesting that NBMPR-P was bound in the form of NBMPR. The influence of NBMPR-P on the binding of NBMPR to HeLa cells is illustrated in Fig. 5. Monolayers were exposed

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[6]As the project progressed, it was discovered that, under the conditions of this experiment, the ^{35}S-content of the medium represented both NBMPR and NBMPR-P and, therefore, that the Table 4 binding "constants" for NBMPR-P were somewhat underestimated.

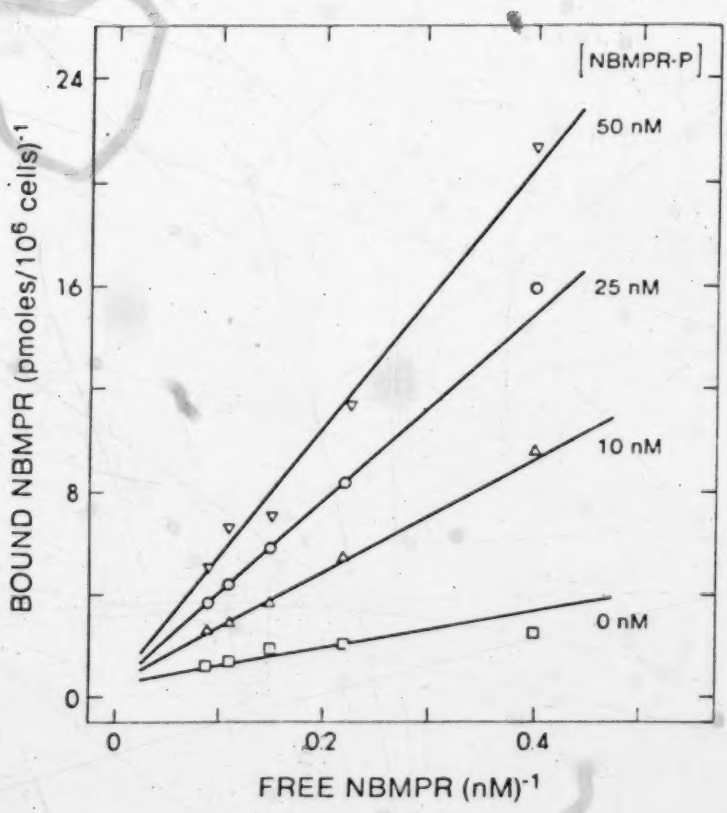

Fig. 5 The influence of NBMPR-P on NBMPR binding

Replicate monolayer cultures were incubated at 20° for 20 min in MEM-T containing various concentrations of [G-^{3}H]NBMPR and nonisotopic NBMPR-P; the monolayers were washed once with ice-cold 0.15 M NaCl solution and the cellular content of ^{3}H was determined by the KOH-Tritosol procedure.

to various concentrations of [G-^{3}H]NBMPR (2 - 10 nM) in the absence and presence of NBMPR-P at concentrations between 10 and 50 nM. The analysis of the binding data so obtained by the double reciprocal plot method (21) indicated that the binding of NBMPR was inhibited competitively by NBMPR-P (Fig. 5). Possibilities suggested by this result were that (i) NBMPR-P competed with NBMPR at the latter's binding sites and (ii) dephosphorylation of NBMPR-P diluted [G-^{3}H]NBMPR. Subsequent results supported the latter possibility.

3. Displacement of the Cell-Bound Substance Derived from NBMPR-P

Previous reports from this laboratory have shown with HeLa cells, the displacement of cell-bound [^{35}S]NBMPR by the tightly bound congener, NBTGR[7] (38), demonstrating the reversible nature of the ligand-binding site interaction. In the present study, it was shown that the cell-bound product derived from exposure of HeLa monolayer cells to [^{35}S]NBMPR-P was displaceable by NBTGR (data not shown) and by NBMPR (Fig. 6, Panel A). The evident similarity with the previous result suggested that the bound product derived from [^{35}S]NBMPR-P was [^{35}S]NBMPR. It is seen in Fig. 6, Panel B, that NBMPR-P also displaced cell-bound [G-^{3}H]NBMPR from HeLa monolayer cells.

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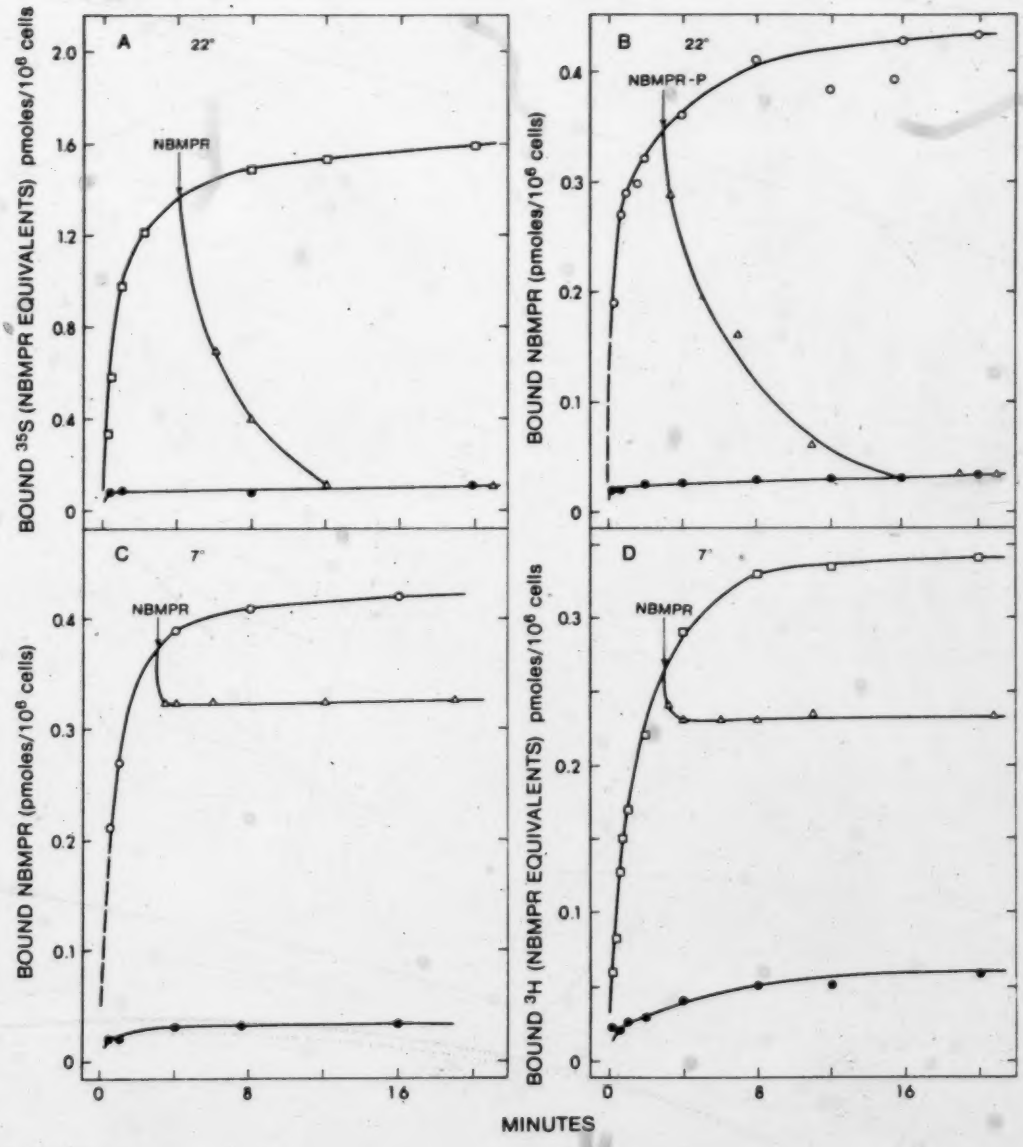
[7]The displacing ligand, NBTGR, was present at a concentration about 1000-fold in excess of the medium concentration of free [^{35}S]NBMPR (38).

Fig. 6. Displacement of cell-associated ^{3}H or ^{35}S derived from [G-^{3}H]NBMPR or [^{35}S]NBMPR-P.

Replicate HeLa monolayer cultures (Panel B) and HeLa cells in suspension (Panel A) were incubated at 22° with MEM-T medium containing 5 nM [G-^{3}H]NBMPR (○) or 5 nM [^{35}S]NBMPR-P (□) in the absence and presence (●) of 5 μM NBTGR to determine site specific binding. In experiments described in Panels C and D, replicate HeLa monolayer cultures were incubated at 7° with MEM-T medium containing 5 nM [G-^{3}H]NBMPR (○) or 5 nM [G-^{3}H]NBMPR-P (□) in the absence and in the presence (●) of 5 μM NBTGR.

Nonisotopic NBMPR or NBMPR-P (final concentration 0.5 μM) was added to the incubation medium at the times indicated (arrow). The cells were washed once with ice-cold 0.15 M NaCl solution and the cellular ^{3}H or ^{35}S determined by the KOH-Tritosol procedure.


A 22°
NBMPR
B 22°
NBMPR-P
C 7°
NBMPR
D 7°
NBMPR
BOUND ^{35}S (NBMPR EQUIVALENTS) pmoles/10^6 cells
BOUND NBMPR (pmoles/10^6 cells)
BOUND NBMPR (pmoles/10^6 cells)
BOUND ^{3}H (NBMPR EQUIVALENTS) pmoles/10^6 cells
MINUTES
0
8
16
0.1
0.2
0.3
0.4
0.8
1.2
1.6
2.0

The experiments of Fig. 6, Panels C and D, were similar to those of Panels A and B in that at 7° NBMPR displaced bound product from [G-^{3}H]NBMPR-P (Panel D) or [G-^{3}H]NBMPR (Panel C), however, displacement in the former proceeded only until about 20% of the bound was released from the cells. The partial displacement of the bound product from NBMPR-P at 7° was similar to that of bound NBMPR from cells suggesting also that NBMPR-P was bound as NBMPR. Together, these results suggested that NBMPR-P was bound in the form of NBMPR.

4. Identity of Cell-Bound ^{3}H Derived from [G-^{3}H]NBMPR-P

Observations discussed above (in particular, (i) the displacement by NBMPR and NBTGR of cell-associated isotopic material derived from labelled NBMPR-P, and (ii) the competitive nature of the inhibition of NBMPR-P binding to HeLa cells by NBMPR and NBTGR), suggesting strongly that the NBMPR-P-derived isotopic material which bound to cells was NBMPR. This idea was tested directly in experiments which showed that the cell-bound radioactivity derived from NBMPR-P labelled with ^{35}S or ^{3}H could be extracted quantitatively from HeLa cells with methanol (Table 2) and was in a form that co-chromatographed with NBMPR on cellulose thin layers in solvents I or II (Fig. 7). NBMPR was the principal cell-associated product derived from NBMPR-P after all of the incubation intervals in the time-course experiment summarized in Fig. 8.

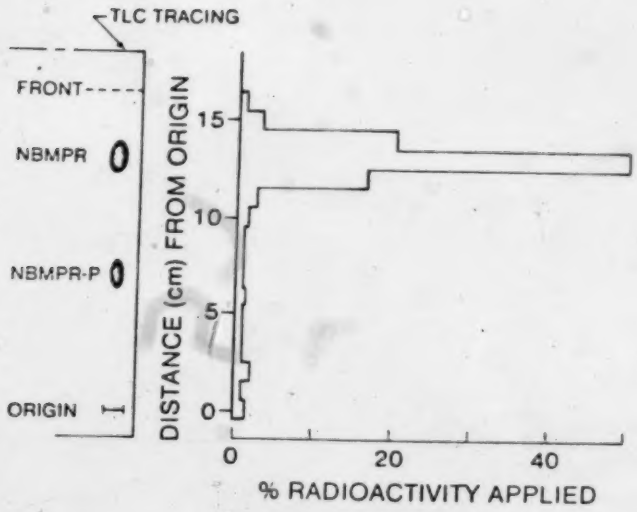


Fig. 7 Formation of NBMPR from NBMPR-P by HeLa cells monolayer

Washed HeLa cell monolayers were incubated with MEM-T medium containing 20 nM [G-^{3}H]NBMPR-P for 10 min. Cells were washed once with ice-cold 0.15 M NaCl solution and the cell-associated ^{3}H extracted with warm methanol; the pooled methanol extracts were dried and then redissolved in 100 µl of 50% methanol. Measured volumes of each extract were applied to cellulose thin layers and developed in solvent III (Table 1). Equivalent volumes were combusted without chromatography to determine the total activity applied.

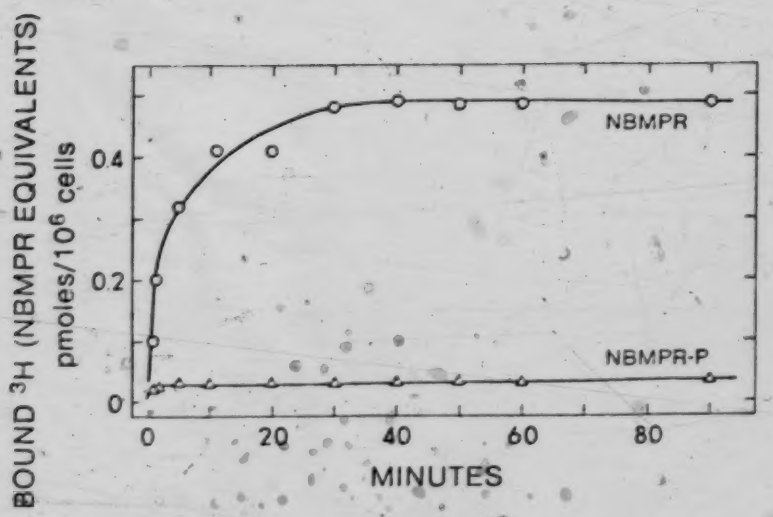


Fig. 8 Time course of the formation of bound NBMPR in HeLa cells incubated with NBMPR-P

Replicate monolayer cultures were washed twice with MEM-T medium and then incubated at 22° in MEM-T medium containing 10 nM [G-^{3}H]NBMPR-P for the indicated intervals; the monolayers were washed once with ice-cold 0.15 M NaCl solution and extracted with methanol. The methanol-extractable material was chromatographed on thin layers of cellulose with NBMPR and NBMPR-P carriers; the ^{3}H accompanying the carriers was determined by the combustion-liquid scintillation method.

5. Discussion

The binding of NBMPR to specific sites on HeLa cells and erythrocytes was apparent in previously reported observations that (i) cell-associated NBMPR was displaced by the congener, NBTGR, and (ii) the cellular content of the "displaceable" NBMPR was a saturable function of the extracellular NBMPR concentration (38,59). By mass law analysis of the specifically bound NBMPR content (i.e., displaceable NBMPR) of HeLa cells and equilibrium concentrations of free NBMPR in the extracellular medium, the dissociation constant of the site-bound ligand was determined, as was the maximum cellular content of ligand. At 20°, these constants were about 10^{-10} M and 10^5 molecules per cell, respectively, for HeLa cells grown in suspension culture. Scatchard plots of these data were linear indicating that the cellular binding sites were of a single type. In the present study which employed HeLa cell monolayer cultures (in contrast to the previous study which employed cells from suspension cultures), dissociation constants were similar to those obtained previously (38) and Scatchard plots were again linear, however, maximum values for the NBMPR content of monolayer cells were 2 - 4 times higher than previously reported (38).

The previously reported inhibition of nucleoside transport by NBMPR-P (44) indicated that this compound, or its dephosphorylation product, interacted with the nucleoside transport mechanism at the NBMPR binding site.

The present study indicated that the dephosphorylation product was the actual inhibitor because the following properties of the cellular ligand derived from NBMPR-P were found similar to those of cell-bound NBMPR: (i) displaceability by NBMPR and NBTGR, (ii) saturability of binding, (iii) binding constants (K_{dissoc} and maximum number of cellular binding sites), and (iv) a single type of high affinity ligand binding site. In addition, binding of the NBMPR-P derived ligand was competitively inhibited by NBMPR and NBTGR. The ligand derived from NBMPR-P was identified as NBMPR by direct isolation: the cell-associated radioactivity derived from ^{35}S- or ^{3}H- labelled NBMPR-P was (i) quantitatively extracted from HeLa cells (as was that bound from exposure to ^{35}S- or ^{3}H- NBMPR) and (ii) almost entirely in the form of NBMPR. It may be noted that methanolic extracts of cells incubated with [G-^{3}H]NBMPR did not contain detectable ^{3}H which co-chromatographed with NBMPR-P suggesting that the latter compound was not formed in HeLa cells.

The dephosphorylation of NBMPR-P, apparent in the above mentioned results, was evidently cell-mediated because binding was assayed in serum-free medium (MEM-T). It was considered likely that this dephosphorylation was due to the ecto-5'-nucleotidase activity known to be a property of the HeLa cell surface (7). The observed displacement by NBMPR-P of cell-bound NBMPR (Fig. 6) and the competitive inhibition by NBMPR-P of NBMPR binding

(Fig. 5) suggested the formation of extracellular NBMPR, a reaction demonstrated subsequently.

B. Involvement of Ecto-5'-Nucleotidase in the Binding of Nitrobenzylthioinosine 5'-Monophosphate to HeLa Cells

It is well recognized that phosphohydrolases specific for nucleoside 5'-phosphates are constituents of the plasma membrane of many animal cells (7,17,27,46,68,73) and such activity has been used as a "marker" in the isolation of plasma membranes.

NBMPR-P has been shown to be an inhibitor of nucleoside transport by HeLa cells of potency comparable to that of NBMPR (44), but it was not apparent whether the nucleotide per se or its dephosphorylation product, NBMPR, was responsible for the inhibition. The present study showed that (i) after exposure of HeLa cells to [G-^{3}H]NBMPR-P, the cell-associated ^{3}H was essentially all in the form of NBMPR, and (ii) that the dephosphorylation of extracellular NBMPR-P by HeLa cells and the extracellular appearance of the reaction product, NBMPR, proceeded under conditions which assured total occupancy of the cellular NBMPR binding sites. These observations suggested that the ecto-5'-nucleotidase activity of HeLa cells (7) was probably responsible for the formation of NBMPR from NBMPR-P, and that this was the basis for the latter's inhibition of nucleoside transport. The ecto-5'-nucleotidase activity is oriented toward the medium and interacts with a variety of nucleoside 5'-monophosphates in the extracellular medium, with reaction products appearing in the medium.

1. Failure of Nitrobenzylthioinosine 5'-Monophosphate to Bind to Erythrocytes

The ability of erythrocytes to bind NBMPR or NBMPR-P was studied in the experiment of Fig. 9 in which mouse or human erythrocytes were incubated with graded concentrations of [G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P in the absence (control) or the presence of a high concentration of NBTGR (3.5 μM). Both types of erythrocyte bound NBMPR and neither cell type was able to bind ^{3}H from [G-^{3}H]NBMPR-P (data not shown). The failure of erythrocytes to bind the ^{3}H from labelled NBMPR-P was attributed to the inability of these cells to dephosphorylate NBMPR-P. The binding data from experiment of Fig. 9 were subjected to mass law analysis by the method of Scatchard (Fig. 10). Similar experiments to determine the number of NBMPR binding sites on erythrocytes from different animal species (human, dog, cat, guinea pig) were performed. The results summarized in Table 5 showed that dog erythrocytes had about one third as many binding sites as human erythrocytes, and that cat and guinea pig erythrocytes had about 9% and 12% as many sites as human cells. The dissociation constants for bound NBMPR were similar. Only a single type of high affinity ligand binding site was apparent in each instance. Neither of the cell types bound ^{3}H from [G-^{3}H]NBMPR-P (data not shown).

The binding of 0.5 nM NBMPR to mouse erythrocyte was inhibited by 100 nM NBMPR-P to a minor extent only, as seen in Fig. 11, but in the presence of the same concentrations

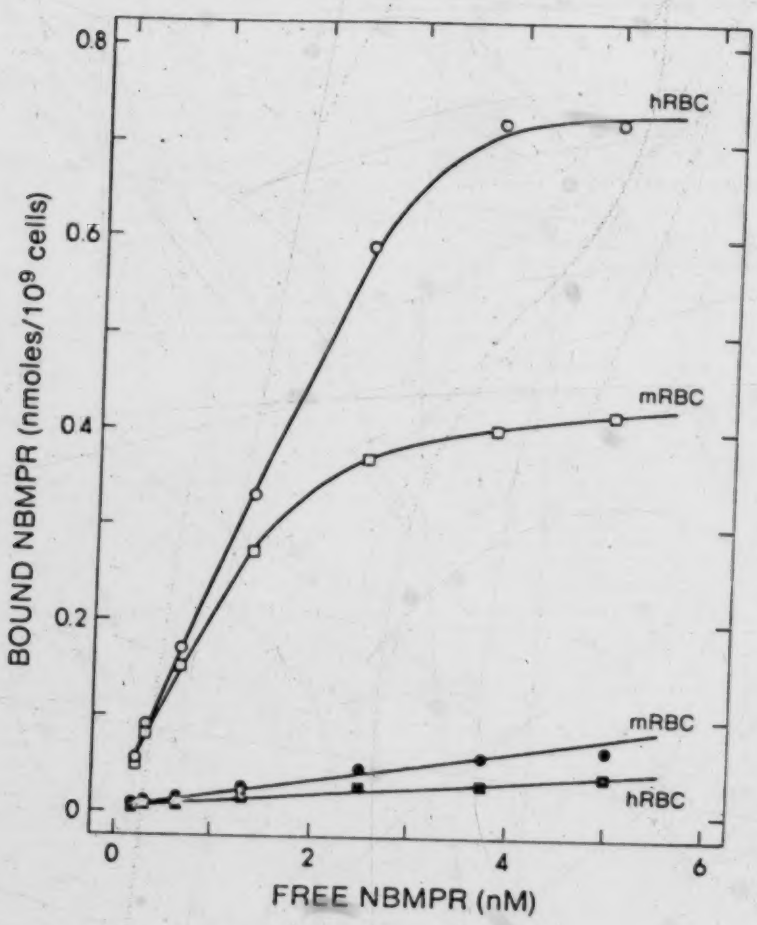


Fig. 9 Binding of NBMPR by human and mouse erythrocytes

Washed erythrocytes from mice (mRBC) and a human (hRBC) were incubated at 22° with graded concentrations of [G-^{3}H]NBMPR without (○,□) or with 3.5 µM NBTGR (●,■) for 30 min. Binding was terminated by centrifugation and the medium content of ^{3}H determined. The amount of ligand taken up by erythrocytes (bound NBMPR) was determined from the difference between the ^{3}H-content of the medium before and after incubation with erythrocytes (free NBMPR).

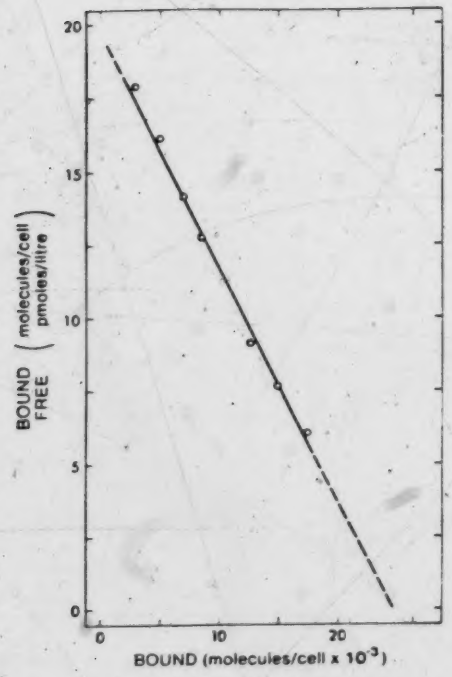


Fig. 10 Binding of NBMPR by mouse erythrocytes

Fig. 9 data for NBMPR bound by mouse erythrocytes are presented here in the form of a mass law (Scatchard) plot (21) from which were obtained the dissociation constant of the bound NBMPR and the maximum number of cellular binding sites.

TABLE 5

Determination of the number of NBMPR binding sites on erythrocytes from various animal species

Washed erythrocytes from different animal species (human, mouse, dog, cat, guinea pig) were incubated at 22° with graded concentrations (0.1 - 10.0 nM) [G-^{3}H]NBMPR without and with 3.5 µM NBTGR to determine specifically bound [G-^{3}H]NBMPR. After incubation for 30 min with occassional stirring, the cells were pelleted and the ^{3}H-content of each supernatant determined in duplicate. The amount of ligand taken up by the erythrocytes in each incubation mixture (bound NBMPR) was determined from the difference between the ^{3}H-content of medium before and after after incubation (free NBMPR). The data obtained were analysed by the method of Scatchard (21) to derive the dissociation constant of the bound NBMPR and the maximum number of cellular binding sites for each cell type.

Table 5, continued:

Source of cells	Binding Characteristics	
	K_{dissoc} ($M \times 10^{-9}$)	Maximum number of molecules bound per cell
Human	1.3	45,000
Mouse	1.2	25,000
Dog	1.2	16,000
Cat	2.5	4,000
Guinea pig	1.4	5,600

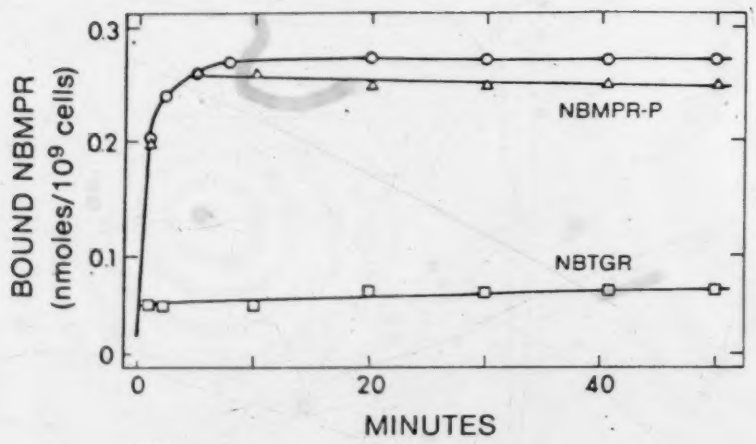


Fig. 11 Influence of NBMPR-P and NBTGR on the time course of NBMPR binding to mouse erythrocytes

Washed erythrocytes were prepared as a set of replicate centrifuge pellets containing 2.0×10^9 cells each. Each cell pellet was suspended in a 2.0 ml portion of MEM-T medium containing 0.5 nM [G-^{3}H]NBMPR without (○) and with 0.1 μM NBMPR-P (△) or 0.1 μM NBTGR (□); suspensions were incubated at 22° for the intervals specified. Incubation intervals began with suspension of pelleted cells and were ended by centrifugation after which the supernatant (medium) fractions were sampled for determination of ^{3}H-content. The cellular content of ^{3}H (bound NBMPR) was determined from the difference between the ^{3}H-content of the medium before and after incubation, the values shown are averages obtained from duplicate incubations.

of NBMPR, was much reduced. The cell content of [G-^{3}H]NBMPR in the presence of 5 μM NBTGR represents NBMPR which is associated nonspecifically with the cells, plus that present in the extracellular water of the cell pellet. A similar concentration of NBMPR-P virtually eliminated the site-specific binding of 0.5 nM NBMPR by HeLa cells (data not shown).

2. Dephosphorylation of Nitrobenzylthioinosine 5'-Monophosphate by HeLa Cells

Preliminary experiments indicated that the dephosphorylation of NBMPR-P which took place in HeLa monolayer cultures was partly attributable to the serum content of the medium and, accordingly, in evaluating the cellular contribution to the dephosphorylation of NBMPR-P, washed monolayers and serum-free medium were employed. The experiment of Fig. 12 showed that the dephosphorylation of NBMPR-P at 5° and 22° was progressive and proceeded with the appearance of the product, NBMPR, in the medium. Dephosphorylation at 5° was slower than at 22°. The dephosphorylation process proceeded with the appearance of NBMPR in the incubation medium well after the cellular NBMPR binding sites were fully occupied by NBMPR (this occured within 10 min of incubation under these conditions at 22°).

The progressive formation of extracellular NBMPR from NBMPR-P was not due to the release of cellular phosphorolytic enzymes into the incubation medium because medium following incubation with HeLa cell monolayers under the

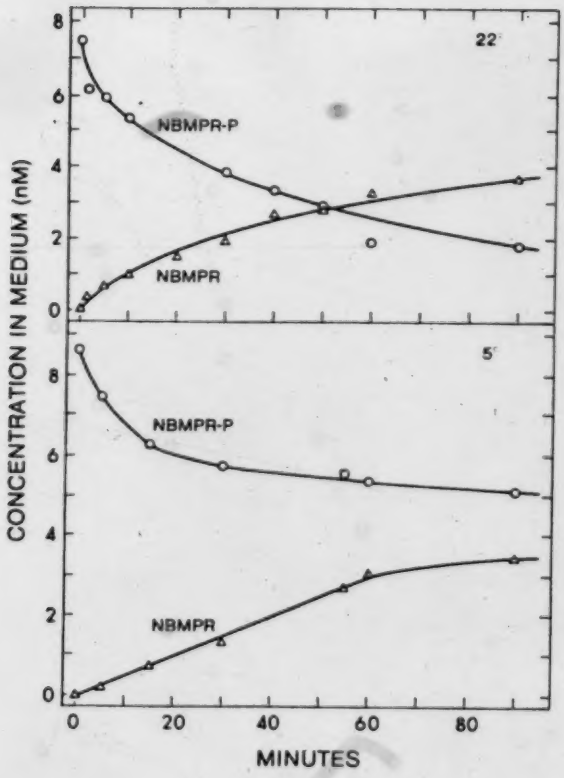


Fig. 12 Dephosphorylation of NBMPR-P by HeLa cell monolayers and extracellular appearance of NBMPR

Replicate monolayer cultures in 8 oz prescription bottles were washed twice with MEM-T medium and incubated with MEM-T medium containing 10 nM [G-^{3}H]NBMPR-P at 22° or 5° for the time intervals indicated. Samples of medium from each incubation were chromatographed with NBMPR and NBMPR-P carriers on cellulose thin layers with solvent I (Table 1). The ^{3}H accompanying the carriers was determined by the combustion-liquid scintillation method.

conditions specified in Fig. 12, did not dephosphorylate NBMPR-P (data not shown). Thus, the production of extracellular NBMPR from NBMPR-P was attributed to the monolayer cells, presumably, to their ecto-5'-nucleotidase activity. In another experiment, medium from HeLa cell suspensions incubated under the conditions specified in Fig. 12 was assayed similarly for the capacity to dephosphorylate NBMPR-P. After 45 min of incubation at 22°, about 30% of the NBMPR-P was dephosphorylated. Thus, a slow loss of enzyme activity from the cells occured; this might derive from sheding of membrane vesicles containing ecto-5'-nucleotidase activity, as has been reported (22,23).

The concept that the ecto-5'-nucleotidase activity of HeLa cells was responsible for the dephosphorylation of NBMPR-P, was tested by determining whether the conversion of free NBMPR-P to cell-bound NBMPR was inhibited by AMP, a substrate for the ecto-enzyme (7,10,69). For the interpretation of experimental work employing this approach, it was necessary to determine whether AMP would inhibit step 2 of the following sequence:

$$\text{NBMPR-P} \xrightarrow{1} \text{NBMPR} \xrightarrow{2} \text{NBMPR.binding site}$$

It is seen in Fig. 13, that the site-specific binding of [G-^{3}H]NBMPR to HeLa cell monolayers was reduced by about 25% in the presence of 1 mM AMP. In a similar experiment, a higher concentration of AMP (2 mM) did not further reduce site-specific binding of NBMPR. The basis for this inhibition is not understood, but it would not appear attributable to competition between AMP-derived adenosine and NBMPR at

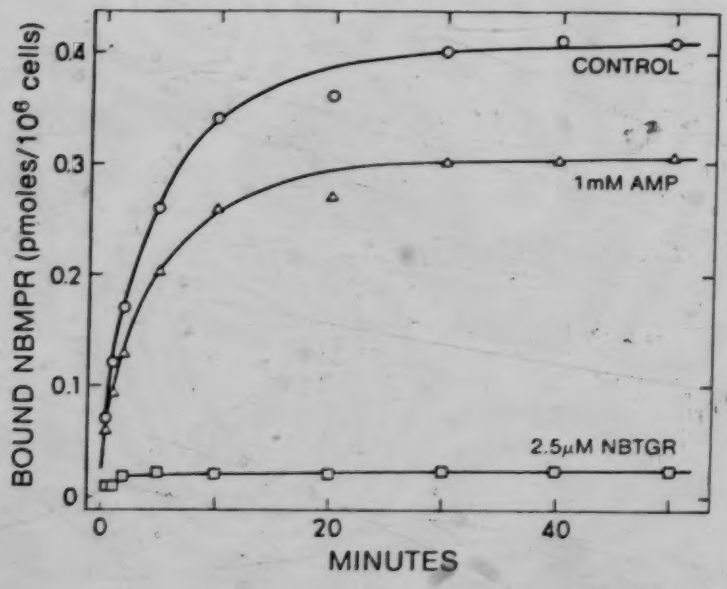


Fig. 13 Inhibition by AMP of site-specific binding of NBMPR by HeLa cell monolayers

Replicate HeLa cells monolayer were incubated at 22° for the indicated intervals of time in MEM-T medium containing 1 nM [G-^{3}H]NBMPR without (O) or with 1 mM AMP (Δ) or with 2.5 μM NBTGR (□). The monolayers were washed once with ice-cold 0.15 M NaCl solution, dissolved in 2.0 ml of 0.5 N KOH and assayed for ^{3}H-content by liquid scintillation counting using the xylene-detergent scintillant.

the latter's binding sites because (i) the concentration of adenosine required to competitively inhibit by 50% the binding of 1.0 nM NBMPR by HeLa cell monolayers is about 400 μM (E. Dahlig, C.E. Cass and A.R.P. Paterson, unpublished results) and (ii) in other experiments, under the conditions specified in Fig. 13, extracellular concentrations of AMP-derived adenosine did not exceed 45 μM[8].

An experiment similar to that of Fig. 13 showed that in the presence of 1 mM AMP, the site-specific binding of NBMPR-P (steps 1 plus 2, see above) was inhibited by about 70% (Fig. 14). Total inhibition of the specific binding of NBMPR-P was achieved by increasing AMP concentration to 2 mM (data not shown). Thus, the large inhibition by AMP of NBMPR binding when the binding substrate was NBMPR-P, relative to that when the binding substrate was NBMPR, appeared to be attributable to AMP inhibition of NBMPR-P dephosphorylation. These results suggested that (i) NBMPR-P binding to HeLa cells proceeded by way of dephosphorylation by ecto-5'-nucleotidase and (ii) the NBMPR site is distinct from

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[8]HeLa monolayers were incubated for 50 min in MEM-T medium containing 1 mM AMP and 2.5 μM NBTGR under the conditions specified in Fig. 13. Measured volumes of medium were spotted on cellulose thin layers and the chromatograms were developed in Solvent III (Table 1). The band accompanying the adenosine control was extracted with water and the adenosine content of the extract determined spectrophotometrically. Adenosine concentrations in the medium, determined in this way, did not exceed 45 μM.

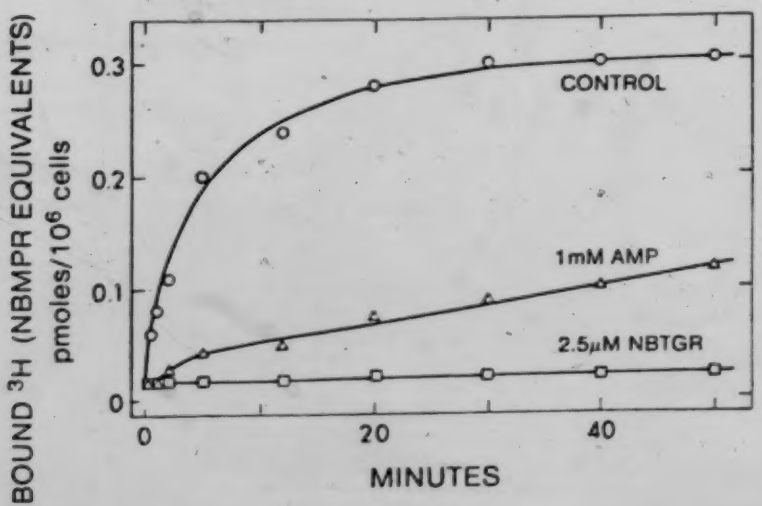

Fig. 14 Inhibition by AMP of binding of NBMPR-P by HeLa cell monolayers

Replicate HeLa monolayer cells were incubated at 22° for the indicated intervals in MEM-T medium containing 1 nM [G-^{3}H]NBMPR-P without (○) or with 1 mM AMP (△) or with 2.5 M NBTGR (□). The monolayers were washed once with ice-cold 0.15 M NaCl solution, dissolved in 2.0 ml of 0.5 N KOH and assayed for ^{3}H-content by liquid scintillation counting using the xylene-detergent scintillant.

the dephosphorylation site. In other experiments (data not shown), it was found that time courses of ^{3}H binding from [G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P by HeLa cell monolayers under conditions identical to those specified in Figs. 13 and 14, were unaffected by the presence of β-glycerophosphate in the medium at concentrations of 2 and 10 mM. Because β-glycerophosphate is not a substrate for, nor inhibitory to ecto-5'-nucleotidase (4), this result is consistent with the notion that activity of the ecto-enzyme is involved in NBMPR-P binding.

3. Kinetics of NBMPR-P Dephosphorylation by HeLa Cell Monolayers

In the experiment of Fig. 12, it was seen that dephosphorylation of NBMPR-P by HeLa monolayer cells proceeded with the appearance of the product, NBMPR, in the incubation medium. These data indicate that the dephosphorylation is a first order process, as might be expected. The rate constant[9] for the disappearance of NBMPR-P was 0.013 sec^{-1}. The dephosphorylation of NBMPR-P by the monolayers under conditions which assured saturation of cellular NBMPR binding sites (Fig. 15) was also a first order reaction process

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[9]Determined from this relationship:

$$\ln \frac{a}{a - x} = kt$$

where a is the initial concentration of NBMPR-P, x is the concentration of NBMPR produced at time, t, and k is the first order rate constant.

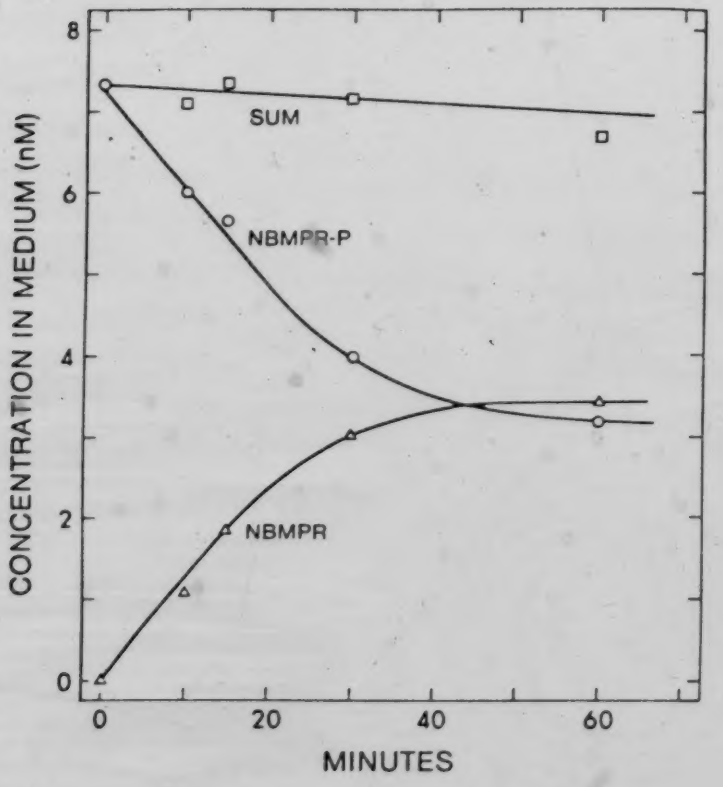


Fig. 15 Dephosphorylation of NBMPR-P by HeLa cell monolayers in the presence of 5 μM NBMPR

Monolayer cultures in 8 oz prescription bottles were washed twice with MEM-T medium and preincubated with 5 μM NBMPR in MEM-T medium for 20 min at 22° ; [G-^{3}H]NBMPR-P (final concentration 10 nM) was added to the medium and incubation at 22° was continued for the indicated intervals. Samples (100 μl) of the medium from each incubation mixture were chromatographed with NBMPR and NBMPR-P carriers on cellulose thin layers with solvent I (Table 1) and the ^{3}H accompanying the carriers was determined by the combustion-liquid scintillation method.

with a similar rate. The data of Fig. 16 indicate that dephosphorylation of NBMPR-P was inhibited by AMP and the extent of that inhibition was related to AMP concentration. While there is considerable scatter in these data, they suggest that the inhibition by AMP was competitive; a replot (Fig. 16 inset) of the reciprocal plot slopes of rate data obtained in the presence of AMP indicated that the K_i value for AMP in this inhibition was 0.19 mM. The apparent participation of AMP in the NBMPR-P dephosphorylation reaction as a competitive substrate indicated that the dephosphorylation was mediated by the HeLa cell ecto-5'-nucleotidase.

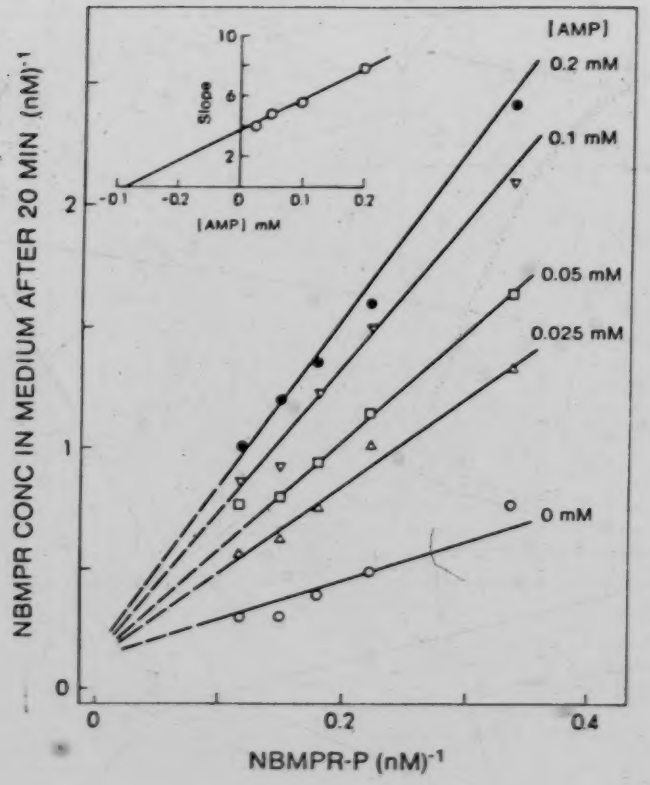
4. Discussion

Previous reports from this laboratory have shown that erythrocytes of human and the mouse possess sites, evidently located on the nucleoside transport elements of the plasma membrane, to which NBMPR binds with high affinity. The existence of these sites was apparent in the present study. The 5'-monophosphate ester of NBMPR did not bind to either type of erythrocyte, neither cell type dephosphorylated the ester, and 100 nM NBMPR-P failed to inhibit the binding of 0.5 nM NBMPR to the high affinity binding sites. These results are consistent with the reported absence of ecto-5'-nucleotidase in human erythrocytes (17) and suggest that NBMPR-P must be dephosphorylated before binding or interaction with the nucleoside transport mechanism is possible.

HeLa cells possess high affinity binding sites for NBMPR with characteristics similar to those of erythrocytes

Fig. 16 Influence of AMP on the rate of NBMPR-P dephosphorylation by HeLa cell monolayers

Monolayer cultures in 8 oz prescription bottles were washed twice with MEM-T medium and preincubated with 5 µM NBMPR in MEM-T medium for 20 min at 22°; [G-^{3}H]NBMPR-P (final concentration 10 nM) was then added to the medium in absence and in presence of various concentrations of AMP and incubation at 22° was continued for 20 min. Samples of assay medium (100 µl) from each incubation mixture were chromatographed with NBMPR and NBMPR-P carriers on cellulose thin layers with solvent I (Table 1) and the ^{3}H accompanying the NBMPR carriers was determined by the combustion-liquid scintillation method. From the replot (inset) of the slopes of the double reciprocal plot, the K_I for the AMP inhibition of NBMPR-P dephosphorylation was determined to be 0.19 mM.


[AMP]
0.2 mM
0.1 mM
0.05 mM
0.025 mM
0 mM
Slope
[AMP] mM
NBMPR CONC IN MEDIUM AFTER 20 MIN (nM)⁻¹
NBMPR-P (nM)⁻¹

(12,38,59), but differ from the latter in possessing ecto-5'-nucleotidase (7). In the present study, the essentially complete inhibition of [G-^{3}H]NBMPR binding (0.5 nM) to HeLa cells in the presence of 100 nM nonisotopic NBMPR-P is attributed to formation of NBMPR by the ecto-5'-nucleotidase activity.

The appearance of enzymatic activity that dephosphorylated NBMPR-P in the medium employed to wash unattached HeLa cells may be explained by the phenomenon of vesicle sheding as suggested by Raz *et al*.(63). Vesicles with 5'-nucleotidase activity have been isolated from mice with ascitic neoplasms and from blood plasma of leukemia patients (2,43).

C. Relation Between Nitrobenzylthioinosine Binding and Ecto-5'-Nucleotidase

NBMPR has been shown to bind to plasma membranes of various cell types (23,59,76), including HeLa cells (38). It was demonstrated in an earlier section (Sec.IV.A.4.) that NBMPR-P is a substrate for the ecto-5'-nucleotidase of HeLa cells and that the product, NBMPR, was bound to cellular sites. Because of an anomalous time course in the binding of NBMPR-P in the presence of NBMPR (see below), it became of interest to explore the relationships between the ecto-5'-nucleotidase and the NBMPR binding site of HeLa cells.

It was observed in the experiment of Fig. 15 that dephosphorylation of NBMPR-P by the ecto-5'-nucleotidase of HeLa monolayer cells proceeded in the presence of 5 μM NBMPR, a concentration that insured saturation of cellular NBMPR binding sites. Under those conditions, dephosphorylation of NBMPR-P was a first order process for which the rate constant was 0.010 sec^{-1}, a value similar to that determined in the absence of NBMPR (0.013 sec^{-1}). The similarity of the rates in the presence and absence of NBMPR suggests that the dephosphorylation sites are separate from and function independently of the NBMPR binding sites. Presuming the validity of this conclusion, it would be expected that (i) [G-^{3}H]NBMPR released by the ecto-5'-nucleotidase from [G-^{3}H]NBMPR-P would be diluted by nonisotopic NBMPR present in the extracellular medium, and (ii) the cellular content

of bound [G-^{3}H]NBMPR derived from either extracellular [G-^{3}H]NBMPR-P or [G-^{3}H]NBMPR under these circumstances should reflect dilution by nonisotopic NBMPR present in the medium. This idea was tested in the experiment summarized in Fig. 17, which showed that a linear relationship existed between the ^{3}H-content of cells and the specific activity of [G-^{3}H]NBMPR under the conditions of this experiment. In this experiment, the same quantities (40 pmoles) of [G-^{3}H]NBMPR were added to replicate monolayer cultures in which the medium contained graded concentrations of nonisotopic NBMPR; the cellular content of [G-^{3}H]NBMPR was assayed after 5 min of incubation at 22°. As would be expected, the labelled NBMPR was diluted by the nonisotopic NBMPR and the cellular ^{3}H-content was proportional to the final specific activity of the [G-^{3}H]NBMPR (See Fig. 17, line A). When the experiment was repeated with [G-^{3}H]NBMPR-P as the labelled substrate, the data of Fig. 17, line B, were obtained. These data indicate that the cellular ^{3}H-content acquired from NBMPR-P under these conditions was directly related to the medium concentration of nonisotopic NBMPR. Thus, the [G-^{3}H]NBMPR formed extracellularly during the 5 min incubation interval mixed freely with extracellular nonisotopic NBMPR. Assuming that (i) in Fig. 17, line A is parallel to line B and (ii) equilibrium between bound and free NBMPR was established within 5 min of incubation, the data of Fig. 17 may be analysed (as illustrated in Appendix 1) to approximate the rate of [G-^{3}H]NBMPR-P

Fig. 17 Binding of [G-^{3}H]NBMPR and [G-^{3}H]NBMPR-P in the presence of nonisotopic NBMPR

Replicate monolayer cultures (4.0 ml) were incubated with MEM-T medium containing 40 pmoles of [G-^{3}H]NBMPR or its 5'-monophosphate for 5 min at 20° without and with additional amounts of nonisotopic NBMPR to achieve the final isotopic dilutions specified. Final specific activities relative to the undiluted binding substrate were real when the latter was NBMPR, but when the substrate was NBMPR-P, the dilutions were hypothetical, calculated assuming that NBMPR-P and NBMPR were equivalent[10]. Observed values of ^{3}H bound (NBMPR equivalents) are plotted against the relative specific activities of the binding substrates, real and hypothetical. After incubation, the monolayers were washed once with ice-cold 0.15 M NaCl solutions, dissolved in 2.0 ml of 0.5 N KOH and assayed for ^{3}H-content by liquid-scintillation counting using the xylene-detergent scintillant.

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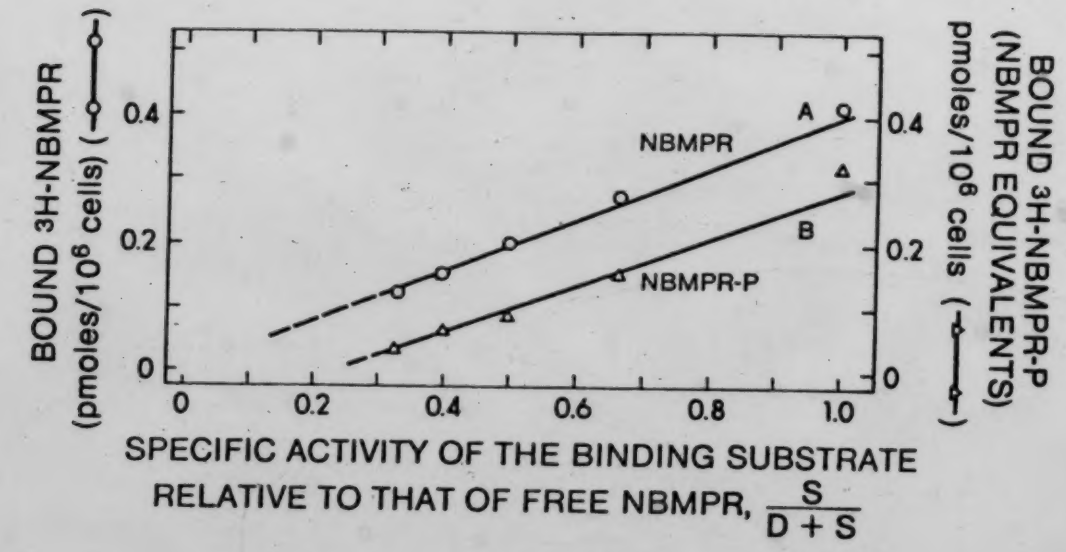
[10]The relative specific activity may also be expresses as $\frac{S}{D + S}$, where S and D are, respectively, the medium concentrations of the isotopic binding substrate and the nonisotopic diluent, NBMPR.


BOUND 3H-NBMPR
(pmoles/10^6 cells) (—o—)
BOUND 3H-NBMPR-P
(NBMPR EQUIVALENTS)
pmoles/10^6 cells (—o—)
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
A
B
NBMPR
NBMPR-P
SPECIFIC ACTIVITY OF THE BINDING SUBSTRATE
RELATIVE TO THAT OF FREE NBMPR, S/(D+S)

dephosphorylation. In this way, it was estimated that in the experiment of Fig. 17, 640 pmole of NBMPR-P was dephosphorylated per min by 10^6 cells. In a separate experiment (Table 6) similar to that of Fig. 17, the rate of NBMPR-P dephosphorylation was found by this method to be 620 pmoles/min/10^6 cells. As noted in Table 6, similar rates of NBMPR-P dephosphorylation were apparent elsewhere in these studies.

The observation that rates of dephosphorylation of NBMPR-P were largely unaffected by concentrations of NBMPR which assured NBMPR saturation of the high affinity binding sites, indicated that the binding of NBMPR-P to those sites did not initiate the dephosphorylation process. Hence, the sites for dephosphorylation and binding were evidently distinct and, accordingly, molecules of the dephosphorylation product, NBMPR, upon release into the medium, would mix by diffusion with NBMPR already present in the medium, but might also have an opportunity to exchange with bound NBMPR if the latter's sites were close or if some local constraint to diffusion existed. The experiment of Fig. 18 was intended to test the idea that initial rates of cellular binding of labelled NBMPR might be affected differently than those of NBMPR-P by the presence of extracellular NBMPR, if the product of NBMPR-P dephosphorylation was released in the immediate vicinity of NBMPR binding sites, or if there existed some constraint to the diffusion of the dephosphorylation product. In the experiment of Fig. 18, replicate monolayers were exposed for short intervals (seconds) to

TABLE 6

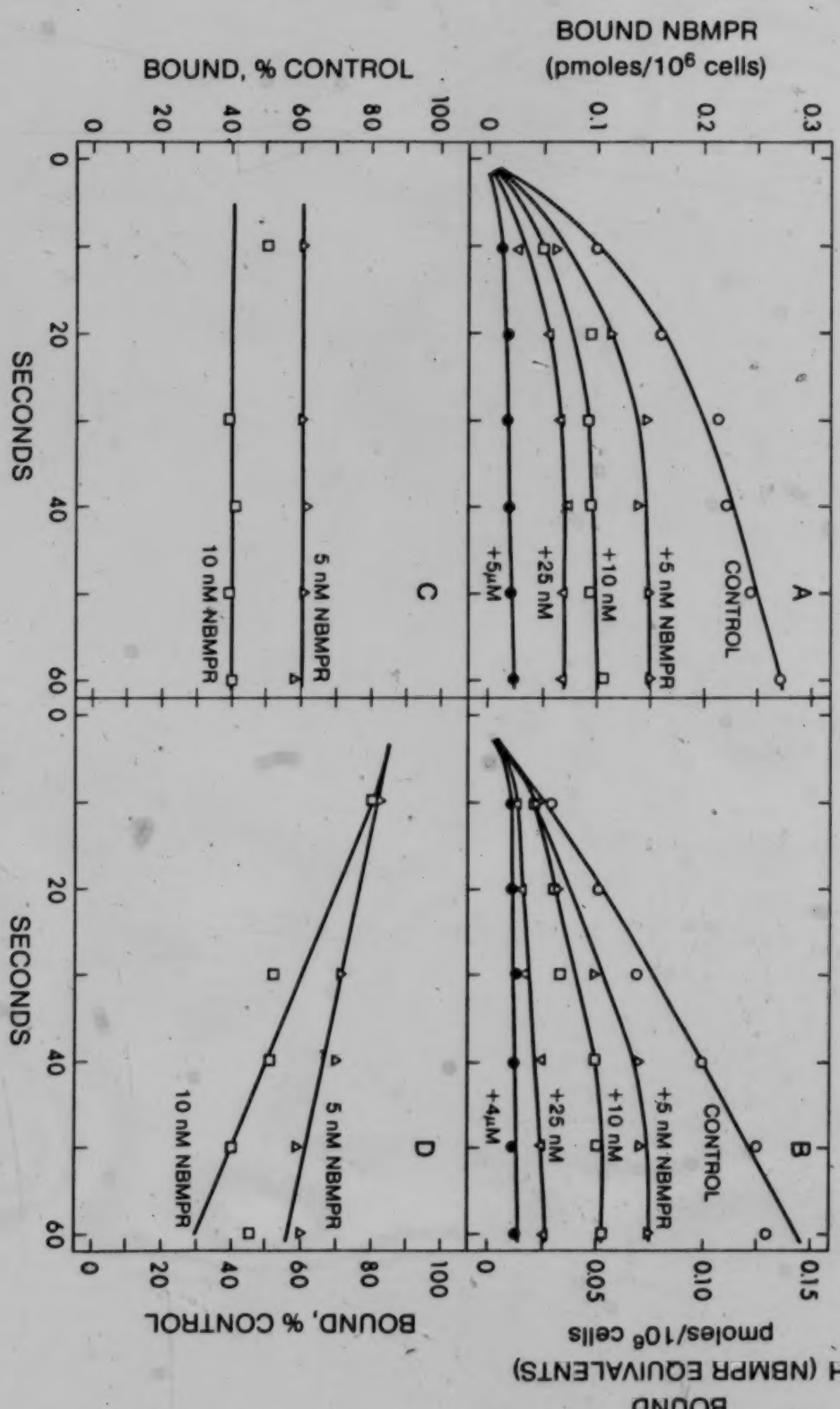
Rates of NBMPR-P dephosphorylation by HeLa cells

In the experiments of Figs. 12 - 17, HeLa monolayer cultures were washed twice with MEM-T medium containing specified concentrations of [G-^{3}H]NBMPR-P with or without unlabelled NBMPR. Aliquots of the incubation medium (100 µl) were removed at intervals and analysed by thin layer chromatography as described in Materials and Methods to determine the amounts of NBMPR product produced by dephosphorylation of the NBMPR-P. The rates reported here were obtained from the figures specified.

Experiment	Rate (pmole/min/10^{6} cells)
Fig. 12	600
Fig. 15	650
Fig. 16	570
Fig. 17	640

Fig. 18 Influence of NBMPR on the initial rates of binding of [G-^{3}H]NBMPR-P and [G-^{3}H]NBMPR

Replicate HeLa cell monolayer cultures were incubated at 22° for the indicated intervals with 5 nM [G-^{3}H]NBMPR (Panel A) or 5 nM [G-^{3}H]NBMPR-P (Panel B) without and with graded concentrations of nonisotopic NBMPR. As in assays to measure initial rates of nucleoside uptake (15), binding intervals were started by rapid immersion of the cell sheets in the ligand-containing medium, 5 sec prior to the end of the interval, medium was removed by suction and the intervals were ended by flooding the cell sheets with ice-cold 0.15 M NaCl solution. The washed monolayers were dissolved in 2.0 ml of 0.5 N KOH and assayed for ^{3}H-content by liquid scintillation counting using the xylene-detergent scintillant. In Panels C and D, the cellular ^{3}H content (in terms of NBMPR equivalents) derived from either substrate in the presence of 5 and 10 nM nonisotopic NBMPR is expressed as a percentage of that in control incubations (absence of nonisotopic NBMPR).


BOUND NBMPR
(pmoles/10^6 cells)
BOUND, % CONTROL
SECONDS
A
B
C
D
CONTROL
+5 nM NBMPR
+10 nM
+25 nM
+5μM
+4μM
5 nM NBMPR
10 nM NBMPR
BOUND
3H (NBMPR EQUIVALENTS)
pmoles/10^6 cells

[G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P in the presence of graded concentrations of nonisotopic NBMPR and, as expected, the cellular ^{3}H-content derived from [G-^{3}H]NBMPR reflected dilution by extracellular, nonisotopic NBMPR. However, when the binding substrate was [G-^{3}H]NBMPR-P, the cellular ^{3}H-content was not a function of the extracellular concentration of nonisotopic NBMPR at the shortest intervals of exposure, suggesting that, to an appreciable extent the dephosphorylation product, [G-^{3}H]NBMPR, was bound before isotopic dilution took place. At longer incubation times, the expected dilution in cell-bound, NBMPR-P derived ^{3}H was observed.

The dilution anomally is illustrated in the data analysis of Fig. 18, Panels C and D. These data show that the cell content of ^{3}H derived from [G-^{3}H]NBMPR, expressed as % of control was independent of time, as would be expect expected. However, when the binding substrate was NBMPR-P, the cellular content of ^{3}H relative to that of controls (cellular ^{3}H derived from [G-^{3}H]NBMPR-P in the absence of external NBMPR) decreased with time, illustrating a basic difference in the initial binding kinetics of NBMPR-P and NBMPR. The result suggests that the dephosphorylation site might be close to the binding site, so that binding of the dephosphorylation product might have occured before substantial diffusion and dilution took place.

V. GENERAL DISCUSSION

NBMPR has been shown to be a potent and specific inhibitor of nucleoside transport in a variety of animal cell lines (12-14,22,23,38,76). NBMPR-P was also shown to be an inhibitor of nucleoside transport in HeLa cells of potency comparable with that of NBMPR (44), but it was not apparent whether the inhibition was attributable to NBMPR-P per se or to NBMPR derived therefrom.

The chemical nature of the inhibitor associated with HeLa cells after incubation with labelled NBMPR-P was established as NBMPR by methanol extraction and chromatographic identification of the isolated compound. The results presented in Tables 2 and 3 demonstrate the quantitative nature of the methanol extraction procedure and the efficiency of the chromatographic isolation methods. The conclusion that NBMPR-P was bound to HeLa cells in the form of dephosphorylation product, NBMPR, was supported by the following observations:

(i) The binding of NBMPR-P was saturable, as was that of NBMPR (Fig. 3).

(ii) The number of NBMPR binding sites per HeLa cell (4.5×10^5 sites per cell) was similar to that for NBMPR-P (3.2×10^5 sites per cell, Fig. 4 and Table 4). Mass law analysis of the binding data by Scatchard method indicated that only a single type of high affinity binding site was apparent with either ligand. As well, the dissociation

constants (about 1.0 x 10^{-10} M) for the bound ligands, as determined from mass law plots, were similar for the two compounds (Table 4).

(iii) The cell-bound product derived from exposure of cells to NBMPR-P was displaceable by NBMPR and NBTGR (Fig. 6).

(iv) Isolation of the cell-bound product (Figs. 7 and 8) revealed only NBMPR. There was no evidence that NBMPR-P was associated with cells.

The dephosphorylation of NBMPR-P was cell-mediated since serum-free medium was used for assays and no evidence was obtained that enzymes leaked into the incubation medium. HeLa cells are known to possess ecto-5'-nucleotidase activity (7). In assessing the involvement of this enzyme in the dephosphorylation of NBMPR-P, it was assumed that NBMPR-P did not penetrate the cell membrane in conformity with the widely-held opinion that nucleotides do not cross intact cell membranes (1,19,45). The product of NBMPR-P dephosphorylation (NBMPR) also appeared in the medium and no evidence was obtained that NBMPR crossed the cell membrane. That the ecto-5'-nucleotidase was responsible for the dephosphorylation of NBMPR-P became apparent when following evidence was considered:

(i) HeLa cells bound ^{3}H from [G-^{3}H]NBMPR-P, but neither human erythrocytes nor erythrocytes from any of the animal species tested did so (Table 5). Erythrocytes of the human are known to possess high affinity binding sites for NBMPR (14), but lack ecto-5'-nucleotidase (17). This observation

also argues for dephosphorylation of NBMPR-P before binding of the product (NBMPR) to the cellular binding sites.

(ii) The data of Fig. 12 showed progressive dephosphorylation of extracellular NBMPR-P by HeLa cell monolayers with the appearance of NBMPR product in the medium. The formation of extracellular NBMPR was not due to the presence in the incubation medium of phosphatases because serum-free MEM-T medium from incubation with HeLa cell monolayers did not dephosphorylate NBMPR-P.

(iii) AMP, a substrate for ecto-5'-nucleotidase (3,7,28,29), competitively inhibited the cleavage of NBMPR-P (Fig. 16). β-Glycerophosphate, a substrate for nonspecific phosphatases (4), did not inhibit dephosphorylation of NBMPR-P. It was concluded that the ecto-5'-nucleotidase was responsible for the dephosphorylation of NBMPR-P.

In the course of this investigation, it was observed that dephosphorylation of NBMPR-P by HeLa cells proceeded in the presence of saturating concentrations of NBMPR (5 μM) in the incubation medium; thus, the possibility of a relationship between the cellular sites for dephosphorylation of NBMPR-P and the binding of NBMPR was investigated. Dephosphorylation of NBMPR-P was not inhibited in the presence of 5 μM NBMPR, indicating that the cleavage sites for NBMPR-P are separate from and function independently of the NBMPR high affinity binding sites. Similar conclusions were also drawn from the experiment which showed that 2 mM AMP caused total inhibition of the site-specific binding of

NBMPR-P to HeLa cells, but had only a minor effect on the binding of NBMPR.

If the dephosphorylation site for NBMPR-P is distinct from the NBMPR high affinity binding site as proposed above, it would be expected that dephosphorylation of NBMPR-P would release NBMPR into the incubation medium which would mix with NBMPR already present in the medium. The experiment of Fig. 18 showed that the initial rate of binding of [G-^{3}H]NBMPR was a function of extracellular concentration of nonisotopic NBMPR, as expected. In contrast, the binding of ^{3}H from [G-^{3}H]NBMPR-P at short intervals of exposure was not a function of the extracellular concentration of nonisotopic NBMPR, suggesting that, to an appreciable extent, the dephosphorylation product, [G-^{3}H]NBMPR, was bound before isotopic dilution took place. At longer incubation times, the expected dilution in cell-bound, NBMPR-P derived ^{3}H was observed.

VI USE OF NBMPR AND ITS 5'-MONOPHOSPHATE AS ADJUNCTIVE AGENTS IN CANCER CHEMOTHERAPY WITH NUCLEOSIDE DRUGS

Studies presently being undertaken by others in this Laboratory indicate the possible use of NBMPR and its 5'-monophosphate as "adjuvants" in chemotherapy with nucleoside drugs. The "adjuvant" role presently being visualized for NBMPR and NBMPR-P is that of affording a possible means of influencing the tissue distribution and pharmacokintics of nucleoside drugs in ways which will improve the therapeutic result. Such effects have been shown and evidently derive from the fact that both compounds are potent inhibitors of nucleoside transport. NBMPR-P has the advantage of being more soluble in water than NBMPR.

Nebularine and many other analogs of the naturally occuring nucleosides are toxic to animals and to cells in culture (6,9,55). In medium containing otherwise toxic concentrations of nebularine, tubercidin, toyocamycin, or sangivamycin, RPMI 6410 cells were shown to proliferate in the presence of NBMPR and NBMPR-P (55,57). Similar results were also reported for L5178Y cells (71). Evidently, the inhibitors of nucleoside transport protected the cells from the cytotoxic nucleosides by blocking transport of the latter into the cells. It is implicit in these observations that the entry into cells of the cytotoxic nucleosides is mediated by the nucleoside transport mechanism.

It has been demonstrated that NBMPR and its 5'-monophosphate will protect mice against potentially lethal doses of several toxic purine nucleosides (55). The protection was effective when the compounds were administered before or simultaneously with the toxic nucleoside analogs. No protection was afforded if NBMPR or NBMPR-P was given 3 hr after the nebularine injection. As well, it has been demonstrated that NBMPR-P will protect leukemic mice from potentially lethal dosage of nebularine and yet allow substantial kill of the leukemic cells, the result being a therapeutic gain through host protection against toxicity of the drug (T.P. Lynch, J.H. Paran and A.R.P. Paterson, unpublished results). The biochemical basis of the in vivo protection also may be a consequence of the ability of the inhibitors to block the transport of the toxic nucleosides into vital organs or tissues. The protection by NBMPR-P would appear to be result of the breakdown of NBMPR-P to NBMPR as demonstrated in the present study. The requirement for conversion of NBMPR-P to NBMPR, may impart some tissue specificity in protection against cytotoxic nucleosides.

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Appendix. Approximation of the rate of [G-^{3}H]NBMPR-P dephosphorylation

The data of Fig. 17 were analysed in the following way. In the abscissa parameter, $\frac{S}{D + S}$, D and S are, respectively, concentrations of diluent (unlabelled NBMPR) and binding substrates ([G-^{3}H]NBMPR or [G-^{3}H]NBMPR-P) in the incubation medium. ^{3}H-Binding data (in NBMPR equivalents) are plotted against the theoretical maximum value of $\frac{S}{D + S}$, that is, as if the binding substrate, [G-^{3}H]NBMPR-P were entirely converted to NBMPR; however, under the conditions of the Fig. 17 experiment, that conversion was only partial. The extent of that conversion may be estimated from the lateral displacement of the A and B plots (Fig.19) as follows. It is assumed that ^{3}H was bound by cells incubated with [G-^{3}H]NBMPR-P in the presence of a particular concentration of nonisotopic NBMPR (such that $\frac{S}{D + S} = X$) as if that incubation had taken place in the presence of [G-^{3}H]NBMPR and nonisotopic NBMPR concentrations such that $\frac{S}{D + S} = Y$. Thus, it may be said that :

$$\frac{S^{NBMPR-P}}{D + S^{NBMPR-P}} = \frac{S^{NBMPR}}{D + S^{NBMPR}} = Y$$

The concentrations of S^{NBMPR} and D are known, hence, the value of $S^{NBMPR-P}$ may be determined. The latter is an approximation only because the actual concentration of NBMPR derived from NBMPR-P changes constantly throughout

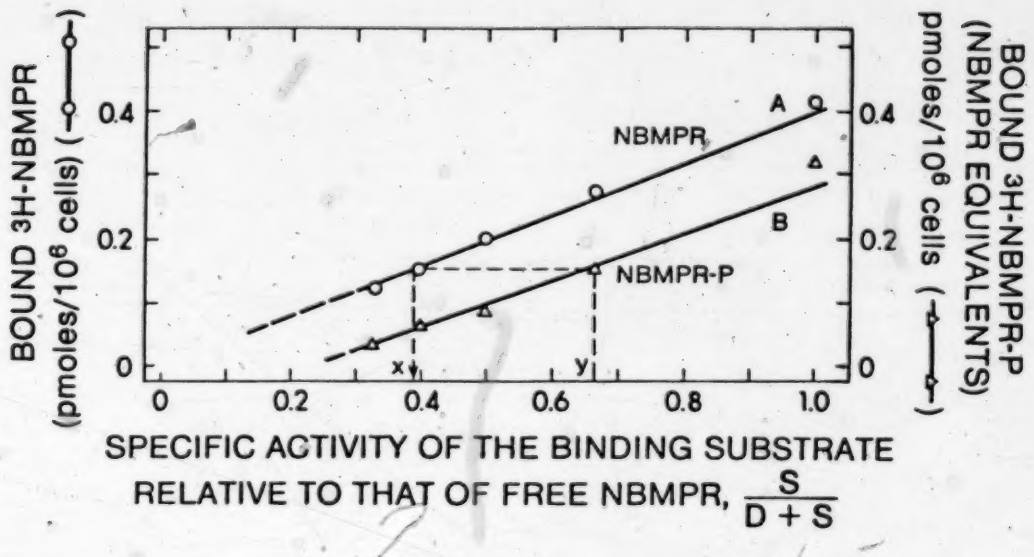

Fig. 19 Approximation of the rate of [G-^{3}H]NBMPR-P dephosphorylation.

the incubation, starting from an initial value of zero. At the NBMPR concentration of 5 nM, equilibrium of bound and free NBMPR is achieved within the incubation period (5 min). This method provides an approximation only of the concentration of NBMPR derived from NBMPR-P during the 5 min incubation.